Isolation and characterization of suppressors of the *prp1* gene, encoding a regulatory component of the pre-catalytic spliceosome in fission yeast

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)
genehmigte
D i s s e r t a t i o n

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eingereicht am: 04.08.2010
mündliche Prüfung (Disputation) am: 09.11.2010

Druckjahr 2010

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurde mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträge vorab veröffentlicht:

Publikationen

Lützelberger M, Bottner CA, Schwelnus W, Zock-Emmenthal S, **Razanau A**, Käufer NF (2010) The N-terminus of Prp1 (Prp6/U5-102 K) is essential for spliceosome activation *in vivo*. Nucleic Acids Res 38(5):1610-22

Tagungsbeiträge

Aleh Razanau, Martin Lützelberger, Ulrike Brandt, Susanne Zock-Emmenthal and Norbert F. Käufer. "Prp1 is a spliceosomal component involved in the activation of pre-catalytic spliceosomes". (**Poster presentation**). Thirteenth Annual Meeting of the RNA Society, Berlin, Germany, 28.07-3.08.2008

Aleh Razanau. "Isolation and characterization of suppressors of the *prp1* gene, encoding a regulatory component of the pre-catalytic spliceosome". (**Oral talk**). International workshop of the Georg-Christoph-Lichtenberg Program of Niedersachsen, Braunschweig, Germany, 25.04 – 26.04.2009

Aleh Razanau, Martin Lützelberger and Norbert F. Käufer. "Characterization of extragenic suppressors of Prp1p encoding a regulatory component of pre-catalytic spliceosomes in fission yeast". (**Poster presentation**). Annual Conference of the German Genetics Society, Cologne, Germany, 16.09-19.09.2009

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1 Summary

Prp1 (U5-102K/Prp6) is a highly conserved spliceosomal protein necessary for the structural integrity of pre-catalytic spliceosomes. The protein consists of multiple HAT (half a TPR) repeats preceded by an N-terminal domain showing no similarity with known motifs. A novel regulatory role of Prp1 has been discovered. Mutations within the N-terminus lead to the accumulation of pre-catalytic spliceosomal particles which consist of snRNPs U1, U2, U5, U4/U6 and contain unspliced pre-mRNA. The mutations in the N-terminus, which prevent splicing to occur, include *in vitro* and *in vivo* identified phosphorylation sites of Prp4 kinase. These sites are highly conserved in the human ortholog U5-102K of Prp1. The results presented here demonstrate that structural integrity of the N-terminus is required to mediate a splicing event, but is not necessary for the assembly of spliceosomes.

Several mutant alleles of *prp1* displaying mutations in various HAT repeats confer temperature sensitivity at the restrictive temperature (35 ºC) causing growth arrest. The growth arrest correlates with the accumulation of unspliced pre-mRNA. The ts allele *prp1-127^{ts}* was used to isolate six extragenic suppressors. Two extragenic suppressors, *spp101-1* and *spp102-1,* were further analysed: *spp102* encodes Spp42/Prp8 (U5-220K), a protein which has been shown to act as a central organizer during activation of a spliceosome; *spp101* encodes Lin1 (U5-52K). Lin1 is not stably associated with Prp1 suggesting transient interaction between these proteins. In a strain expressing *prp1-127^{ts}* the steady state level of the mutant Prp1$_{G705D}$ protein is visibly lower when compared with Prp1^{wt} in a wild-type strain at 35 ºC. The expression of the mutant Lin1$_{D167N}$ protein in the suppressor strain (*spp101-1/lin1-1 prp1-127^{ts}*) does not lead to the stabilization of Prp1$_{G705D}$ at 35 °C suggesting that the mechanism of suppression is not due to the stabilization of Prp1 by Lin1. The mutant Prp1$_{G705D}$ still able to associate with pre-catalytic spliceosomes prevents their activation at the restrictive temperature. Expression of the suppressor gene *spp101-1/lin1-1*, however, allows activation of those pre-catalytic spliceosomes associated with Prp1$_{G705D}$. Therefore, it is conceivable that Lin1$_{D167N}$ improves the interactions of other spliceosomal proteins with Prp1$_{G705D}$ thus operating as a chaperone. The data obtained are consistent with the idea that Lin1 may act as an effector which helps Prp1 to adopt a conformational state required to promote the activation of the spliceosome.

2 Introduction

2.1 Pre-mRNA splicing *in vitro*

Pre-mRNA splicing takes place in a snRNA-protein complex called the spliceosome. Two transesterification reactions, most likely catalyzed by the snRNA parts of the spliceosomal machinery have been shown to mediate the cut at the 5' splice site leading to a 5'-2'-lariat intron-exon 2 molecule and a free 3'-OH end of exon1. This end reacts with the 3' splice site of the lariat-exon 2 intermediate and leads to the splicing of exon 1 with exon 2, whereas the intron is released as a lariat structure. These reactions have been elucidated using *in vitro* systems of HeLa cells and budding yeast, demonstrating that the nature of the splicing reaction is universal in eukaryotes. Extensive studies with these two *in vitro* systems over the last 25 years led to the suggestion of a stepwise assembly pathway of sub-spliceosomal particles resulting in a complex containing the five snRNAs U1, U2, U5 and base paired U4/U6 associated with more than 100 proteins (Reviews: Jurica and Moore, 2003; Wahl *et al*. 2009). The snRNAs associated with proteins are called snRNPs (small nuclear ribonucleoprotein particles). The sub-spliceosomal particles have been defined biochemically by the isolation and characterization of the particles from both *in vitro* systems and then were tested functionally by adding them back into the system containing a model pre-mRNA to measure splicing activity. Based on these *in vitro* studies, it has been suggested that the pre-mRNA is first bound by the snRNP U1 through complementary basepairing with the 5'-splice site, followed by the snRNP U2 recognizing the region around the 3'-splice site including the branchpoint sequence. Then, a tri-snRNP consisting of U5.U4/U6, in which the snRNAs U4 and U6 are basepaired (U4/U6), is recruited. This particle containing U1.U2.U5.U4/U6 represents a splicing competent pre-catalytic spliceosome and specifies the boundaries of the intron to be spliced out (Figure 2.1).

In the next step, the pre-catalytic spliceosome is activated. Activation induces extensive rearrangements of the snRNPs forming the catalytic site consisting of the snRNAs U2/U6 and U5 (Figure 2.1).

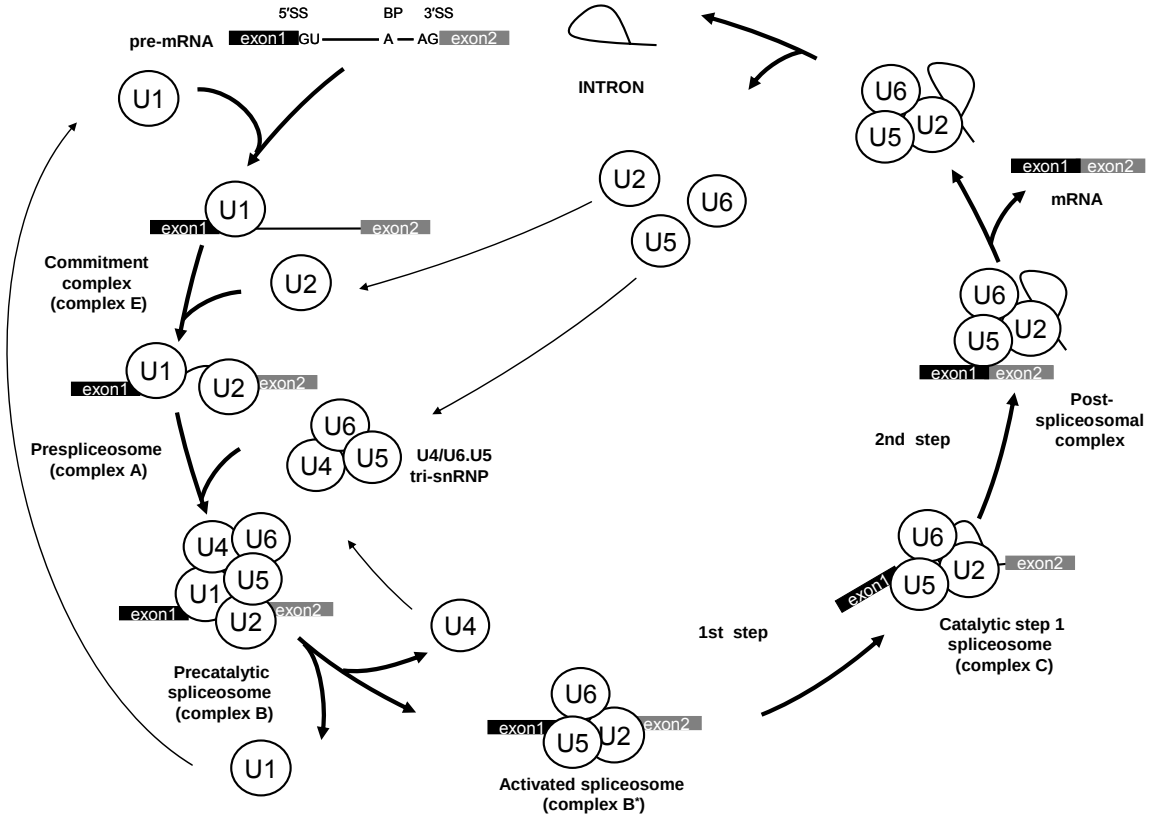

Figure 2.1 Assembly and disassembly cycle of the spliceosome. The stepwise interaction of the spliceosomal snRNPs (circles marked with U1, U2, U4, U5 and U6) during the removal of an intron from a pre-mRNA containing two exons (black and gray) is depicted. 5′SS: 5′ splice site; 3′SS: 3′ splice site; BP: branch point; 1st step: the first transesterification step; 2nd step: the second transesterification step (modified from Wahl *et al*. 2009).

It is not known what activates a pre-catalytic spliceosome *in vitro*. However, several components playing a key role in the activation process have been identified. Prp8/Spp42, a protein of 270 kDa associated with snRNP U5, is discussed as the central organizer of the rearrangements. It appears that this protein provides the surface area for all the rearrangements to form the catalytic core and is possibly also actively involved in these rearrangements providing a chaperone function. However, for this function clear evidence is still lacking (Abelson, 2008; Ritchie *et al*. 2008; Bellare *et al*. 2008; Newman and Nagai 2010). Another key component in the activation process is Brr2, a protein of 220 kDa which is also associated with snRNP U5. Brr2 encodes an ATP-dependent RNA helicase of the DEAD-box family and has been shown to unwind U4/U6. Thus, to rearrange a pre-catalytic spliceosome containing the five snRNPs U1.U2.U5.U4/U6 *in vitro* into a catalytically active spliceosome containing U2.U6.U5, Brr2 is most likely involved in unwinding the base paired regions between U4/U6 to remove U4 during activation (Brow 2009; Maeder *et al*. 2009). Another DEAD-box helicase family member seems to be

involved in unwinding basepaired regions between the 5' splice site of the pre-mRNA and U1 snRNA to remove U1 during activation (Chen *et al*. 2001).

2.2 Pre-mRNA splicing *in vivo*

How a spliceosome is assembled and activated *in vivo* is still completely unknown, but is a matter of intense debate and speculation (Rino and Carmo-Fonseca, 2009). The textbook Molecular Biology of the Cell (2008, fifth Edition, Chapter 6, p350) outlines this debate in one sentence:

„However, many scientists believe that, inside the cell, the spliceosome is a preexisting, loose assembly of all the components- capturing, splicing and releasing RNA as a coordinated unit, and undergoing extensive rearrangements each time a splice is made." (2008, fifth edition, Chapter 6, p 350).

As a matter of fact, it was Joseph and Ruth Sperling, who discovered and provided evidence that in mammalian cells *in vivo* so called supraspliceosomes exist. It was shown that a supraspliceosome consists of four pre-assembled 60S core spliceosomes containing the snRNPs U1, U2, U5. and U4/U6. Moreover, it was suggested that a holospliceosome, consisting of the five snRNPs U1, U2, U5, U4/U6, is recruited to the pre-mRNA during transcription, then the exons are spliced by looping out the intron (Azubel *et al*. 2004; Azubel *et al*. 2006; Sperling *et al*. 2008). As stated by the authors: „The supraspliceosome model allows the recognition of an intron as an entity, rather than recognizing its boundaries in a consecutive set of interactions with splicing factors. The synergistic effect of simultaneous recognition of multiple sites is likely to increase the accuracy of the splicing reaction. Furthermore, the supraspliceosome presents a platform on which splice junctions could be checked prior to intron excision, thereby enhancing the fidelity of the splicing reaction. In conclusion, the model of the supraspliceosome enables mechanistic and temporal compartmentalization of the splicing reaction, because each native spliceosome can process one intron at a time. "Azubel *et al*. J. Mol. Biol. (2006) 356, 955-966, p 963.

The isolation and characterization of supraspliceosomes from mammalian and vertebrate cells confirm the existence of supraspliceosomes (polyspliceosomes) *in vivo* (Chen *et al*. 2007). Experiments using chromatin immunoprecipitation (ChIP) to analyse loading of spliceosomal components onto pre-mRNA during transcription provide support for the model that a holospliceosome (penta-snRNP) may be loaded onto pre-mRNA (Pandya-Jones and Black, 2010, Chen *et al*. 2007, Listermann *et al*. 2006).

It was the isolation and characterization of a spliceosomal complex from the budding yeast *Saccharomyces cerevisiae* containing the five snRNAs and most of the known snRNP proteins which led to the suggestion that a penta-snRNP (holospliceosome) may assemble independently of pre-mRNA (Stevens *et al*. 2002). ChIP analysis to determine whether pre-mRNA splicing in *S. cerevisiae* is co-transcriptional and whether there is a sequential order of events in forming a splicing competent spliceosome *in vivo* suggest that in *S. cerevisiae* the U1 snRNP recognizes pre-mRNA first. It could not be determined in these experiments whether after the interaction of U1 with pre-mRNA in the next step U2 and then a pre-formed tri-snRNP U4/U6.U5 or a tetra-snRNP U2.U4/U6.U5 is recruited to a U1 snRNP-pre-mRNA complex (Görnemann *et al*. 2005; Lacadie and Rosbash, 2005; Nilsen, 2005). Remarkably, in these series of experiments it was found out that in *S. cerevisiae* ≤ 10 % of the intron containing genes are spliced co-transcriptionally (Tardiff *et al*. 2006). The total number of genes containing introns in *S. cerevisiae* is 253. In other words, 4.3 % of the genes contain one intron with an average length of 257 nts, that is, the number of co-transcriptionally spliced genes is almost negligible (Kupfer *et al*. 2004).

2.3 Pre-mRNA splicing in *S. pombe*

Schizosaccharomyces pombe contains 4730 introns, that is, 45 % of its genes have introns. Many genes display multiple introns and the introns, with a few exceptions, are very short with an average length of 107 nt (Kupfer *et al*. 2004; Wood *et al*. 2002). Recently, the group of Jose Ayte presented the first example of true regulation of pre-mRNA splicing in fission yeast. The *rem1* gene encoding a meiotic cyclin is only expressed when a 87 nt intron is spliced out. Splicing of *rem1*-pre-mRNA is dependent on the meiosis specific transcription factor Mei4. Mei4 regulates in meiosis the transcription of *rem1* and recruits the splicing machinery to the transcribed locus (Malapeira *et al*. 2005; Moldon *et al*. 2008). Meanwhile, several genes have been found which are regulated by pre-mRNA splicing during meiosis (J. Ayte, personal communication), however, it is obvious that in general removal of introns in fission yeast occurs constitutively by default. Nothing is known how introns are recognized and how splicing is initiated *in vivo*.

Inspite intensive efforts over many years, all attempts to study *in vitro* assembly of spliceosomal particles with *S. pombe* cell-free extracts failed. These investigations revealed, however, that whole-cell extract (WCE) of *S. pombe* contains only minute amounts of tri-snRNP U4/U6.U5 (Huang *et al*. 2002).

Remarkably, it was shown that *S. pombe in vivo* splices correctly the 66 nt intron of the

SV40 small t antigen transcript, which is not recognized as an intron by *S. cerevisiae* (Käufer *et al*. 1985). Further studies exploring intron recognition in *S. pombe* revealed that artificial introns introduced into naturally intronless genes are correctly recognized and efficiently spliced as long as these introns display proper 5'- and 3' splice sites, and also notably, as long as the size of the introns is ≤ 100 nt (Gatermann *et al*. 1989). In addition, in a study investigating recognition of introns with authentic intron containing genes, it was found that *S. pombe* favors to splice between 5'- and 3'- splice sites which excise the smallest intron possible (Romfo *et al*. 2000). These observations suggest that the splicing machinery recognizes an intron by placing the splicing machinery across the intron.

In the nineties a hypothesis for intron recognition in vertebrates, called exon definition model, was put forward (Berget, 1995). This model states that an exon bordered by intron A and intron B (intron A- 3'- exon Z -5'-intron B) is involved in intron A and intron B recognition before pre-spliceosomal assembly. The snRNP U1 binds to the 5'-intron B splice site and interacts with the splicing components U2AF/SF1 which bind to the 3'-splice site of intron A. Thus marking intron borders of one exon and forming a complex across the exon which involves the serine/arginine-rich proteins (SR) (Figure 2.2).

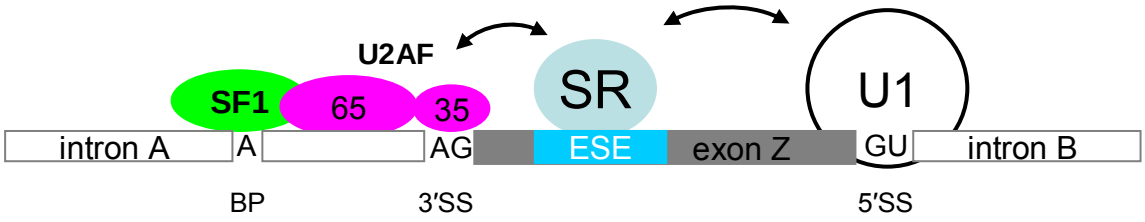

Figure 2.2 Cross-exon splicing complexes form during the earliest stage of spliceosome assembly. An SR protein is depicted as interacting with an exonic splicing enhancer (ESE [blue box]). The U1 spliceosomal snRNPs, the two subunits of the U2 auxilliary factor (U2AF), U2AF65 and U2AF35, and SF1 (also known as the branch-point binding protein [BBP]) are shown interacting with the splice sites flanking the exon Z (gray boxes). 5′SS: 5′ splice site of the intron B (white box to the right); 3′SS: 3′ splice site of the intron A (white boxes to the left); BP: branch point of the intron A (modified from Wahl *et al*. 2009). Additional explanations see in the text.

The SR protein family consists of nine members and the proteins known as splicing regulators involved in constitutive and differential splicing bind to exonic and intronic sequences. There are no SR proteins in *S. cerevisiae*, but there are two in *S. pombe* (Schellenberg *et al*. 2008; Ram and Ast 2007; Collins and Penny, 2005; Lützelberger *et al*. 1999; Groß *et al*.1998). However, as discussed above, the short introns in *S. pombe* are most likely not recognized by an exon definition mechanism, rather, the information to be

recognized as an intron is inherent to the intronic sequence. This has been termed intron definition. Other species such as *Caenorhabditis elegans*, *Drosophila melanogaster* and plants, where there are small as well as large introns, appear to have both intron and exon definition mechanisms in operation (Collins and Penny, 2005; Ram and Ast, 2007).

2.4 Spliceosomal particles in *S. pombe*

Three *in vivo* derived large spliceosomal complexes from *S. pombe* have been isolated and analyzed. First, the group of Norbert F. Käufer isolated and analyzed the U1 particle. Unexpectedly, the U1 snRNP exists as a multimeric 45S particle containing exclusively snRNA U1 associated with the seven Sm core proteins and nine U1 specific proteins (Newo *et al*. 2007). Second, based on the analysis of a 35S complex containing the snRNAs U2, U5 and U6, it was shown that it consists of a mixture of activated- and post-catalytic spliceosomal complexes. These spliceosomal subcomplexes did not contain the *bona fide* splicing factors Prp1 and Prp31 (McDonald *et al*. 1999; Ohi *et al*. 2002; Ohi *et al*. 2005; Ohi *et al*. 2007). Third, a complex associated with Prp1 and Prp31 has been isolated and proteomic analysis of the complex revealed that it contains proteins expected in a spliceosomal sub-complex containing the four snRNAs U2, U5 and base paired U4/U6. This complex might be called a tetra-snRNP (Carnahan *et al*. 2005). Prp1 and Prp31 are found associated with large spliceosomal complexes co-sedimenting in a glycerol gradient in the range of 30–60S. The spliceosomal complexes sedimenting in this range appear to be a mixture of pre-catalytic spliceosomal particles containing the four snRNAs U2, U5 and U4/U6 (tetra-snRNP) as well as complexes containing the five snRNAs, including U1 (penta-snRNP). These results indicate that Prp1 and Prp31 are spliceosomal proteins associated with pre-catalytic spliceosomal particles, but not with post-catalytic complexes (Bottner *et al*. 2005).

2.5 Role of Prp4 kinase in pre-mRNA splicing in *S. pombe*

Over the last 15 years the group of N.F. Käufer was investigating the role of Prp4 kinase in pre-mRNA splicing using *S. pombe* as a model organism. *S. cerevisiae*, in fact, all hemiascomycetes do not contain a counterpart of Prp4 kinase, but all other organisms whose genome is known do contain a Prp4 kinase (Kuhn and Käufer, 2003; Kuhn and Käufer, 2004; Bon *et al*. 2003). After demonstrating that the spliceosomal protein Prp1 in *S. pombe*, whose orthologue in *S. cerevisiae* and in HeLa cells is called Prp6 and U5-102K, respectively, is a physiological substrate of Prp4 kinase it was proposed: „Altogether, the

results discussed above suggest the intriguing hypothesis that Prp4p is a signaling kinase whose activity controls the formation of active spliceosomes. The signal is triggered by phosphorylating Prp1 and, based on our current knowledge, this could take effect on two levels. First, it might be at the level of spliceosome assembly. Second, phosphorylation of Prp1p might be involved in switching a spliceosome into an active state by inducing the rearrangements of the snRNAs and the pre-mRNA substrate to fold into a catalytic center. Then, phosphorylation of Prp1 would occur after spliceosome assembly. These two hypotheses, which can be experimentally tested, are not mutually exclusive.“ (Kuhn and Käufer 2003, p 247).

Very recently, the phosphorylation sites in Prp1 by Prp4 kinase have been determined. It was found that the N-terminal domain of Prp1 is phosphorylated at several sites. These sites are also phosphorylated in the human orthologue of Prp1 (U5-102K), whereas the N-terminus of Prp6 of *S. cerevisiae* is completely different in sequence in this region (Lützelberger *et al*. 2010). The phosphorylation sites of huPRP4 kinase in human Prp1 (huPrp6/U5-102K) have now been confirmed (Schneider *et al* 2010). The results presented in our publication demonstrate that structural integrity of the N-terminus of Prp1 is required to mediate a splicing event, but is not necessary for the assembly of spliceosomes. The deletion of the N-terminal region containing the phosphorylation sites leads to the accumulation of pre-catalytic spliceosomal complexes containing the five snRNAs U1, U2, U5 and U4/U6 and pre-mRNAs. Based on these results and results of our ongoing research, it is reasonable to suggest that phosphorylation of Prp1 by Prp4 kinase plays a regulatory role on a pre-catalytic level — either the phosphorylation by Prp4 kinase is part of a mechanism that signals that an intron is occupied by a splicing competent spliceosome in a sense of quality control and/or the phosphorylation is directly involved in inducing the rearrangements for catalysis.

2.6 Aims

The main goal of this project is to unravel the regulatory role of Prp1 in the splicing process. For this purpose the molecular consequences of the expression of specific deletion mutations in the N-terminal domain of Prp1 were investigated. The mutations include the phosphorylation sites of Prp4 kinase. The expression of these mutations leads to the stalling of pre-catalytic complexes containing pre-mRNA.

Further, I will present the molecular isolation and characterization of suppressors of the *prp1-127*^{ts} allele. Six different extragenic suppressor genes of this allele were available (K. Böhme, 2003, Diploma thesis, Institute of Genetics, TU Braunschweig). The molecular isolation and characterization of two of the components which genetically interact with Prp1 will help to further elucidate the precise regulatory role of Prp1 in the splicing process.

Results

3.1 *prp* mutants

The research groups of David Frendewey, Judy Potashkin, Tokio Tani and Norbert F. Käufer made a collection of temperature sensitive (ts) mutants of *S. pombe* whose common phenotype, besides arresting growth at the restrictive temperature (35 °C), is the accumulation of pre-mRNAs of intron containing genes. These genes were termed *prp1*^{ts}-*prp14*^{ts} (*p*re-m*R*NA-*p*rocessing; for example: *prp1-127*^{ts} Figure 3.1 A and B; Potashkin *et al.* 1989; Rosenberg *et al.* 1991; Urushiyama *et al.* 1996; Urushiyama *et al.* 1997; Potashkin *et al.* 1998).

3.2 The genes *prp1*, *prp4* and *prp31*

The isolation of several *prp* genes by complementation of the temperature sensitive phenotype of the respective mutant alleles revealed *bona fide* evolutionary conserved spliceosomal components. These include Prp1, a highly structured protein containing eleven HAT (Half a TPR) domains (Figure 3.1 A) and the snRNA U4 encoded by *prp13*^{ts} (Urushiyama *et al*. 1996; Urushiyama *et al*. 1997). These spliceosomal components have orthologues in *S. cerevisiae* and mammals. However, there is one exception. The *prp4* gene encoding a serine/threonine kinase has no counterpart in the budding yeast *S. cerevisiae*, but is found in all other (sequenced) eukaryotic organisms. Interestingly, the kinase is not found in all Hemiascomycetes to which *S. cerevisiae* belongs (Bon *et al*. 2003).

Almost nothing is known about the function of Prp4 kinase in the pre-mRNA splicing process. Over the last decade the group of N. F. Käufer established a genetic interaction map of Prp4 kinase identifying suppressors of the *prp4-73*^{ts} allele and probing for synthetic lethal interactions with *prp4-73*^{ts}. Mutant alleles of *spp41/brr2* and *spp42/prp8* have been identified as extragenic suppressors of *prp4-73*^{ts} (Schmidt *et al*. 1999; Schwelnus *et al*. 2001; Kuhn and Käufer, 2003; Bottner *et al*. 2005). Spp42/Prp8 encodes an evolutionary highly conserved *bona fide* spliceosomal protein of 270 kDa which has been shown to play a central role in spliceosome activation. In particular, this protein has been discussed to serve as an interacting platform for spliceosomal proteins involved in activating the pre-catalytic spliceosome (Kuhn *et al.* 2002; Abelson 2008). The temperature sensitive alleles *prp1-4*^{ts} and *prp1-127*^{ts} (Figure 3.1 A) show synthetic lethality with *prp4-73*^{ts}. That is, cells containing one of these *prp1* alleles in a genetic background with *prp4-73*^{ts} do not grow even at the permissive temperature of 25 °C. Further characterization of this genetic

interaction using molecular methods led to the discovery that Prp1 is a physiological substrate of Prp4 kinase (Schwelnus *et al*. 2001). This result was confirmed in the mammalian system. The mammalian orthologue of Prp4 kinase is called PRP4K. The orthologue of *S. pombe* Prp1 in mammals is called U5-102K or, unfortunately, PRP6 (Dellaire *et al*. 2002).

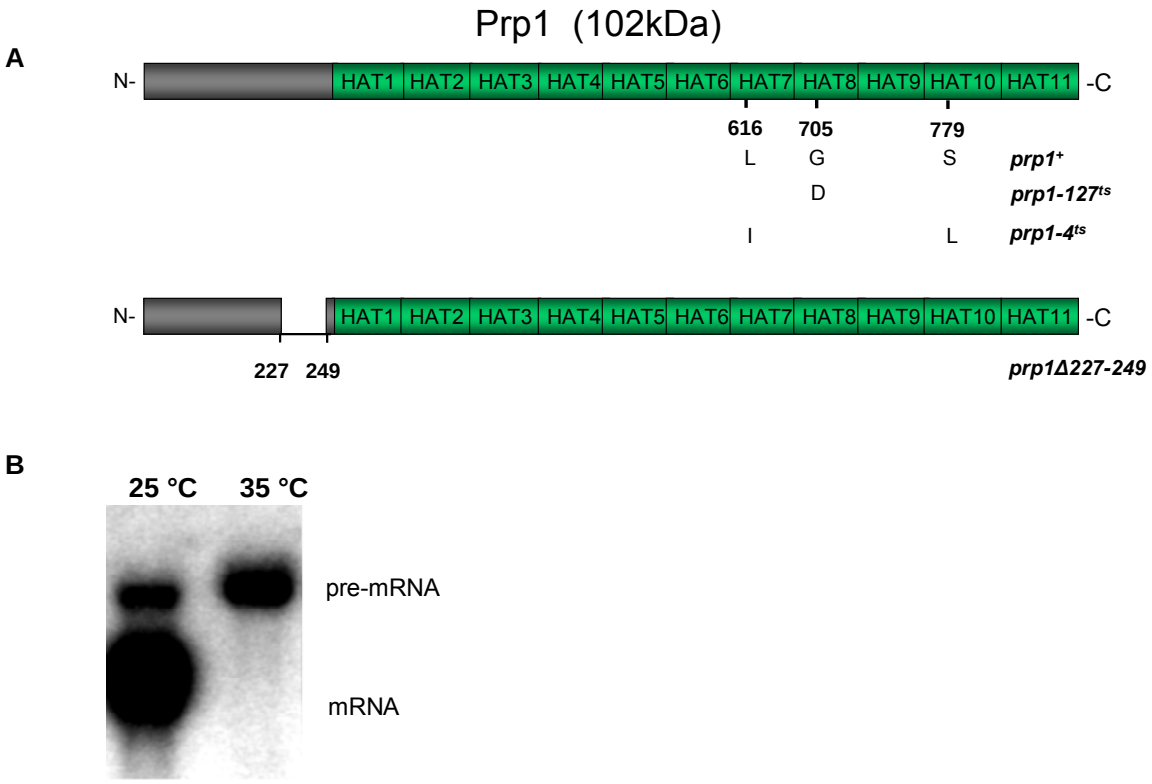

Figure 3.1 A Prp1 showing 11 HAT domains (Half a TPR) at the C-terminus and no known motifs at the N-terminus. The temperature sensitive (ts) allele *prp1-127^{ts}* contains a pointmutation which changes a glycine residue (G) to an aspartic acid (D) at amino acid position 705 in HAT 8. The ts allele *prp1-4^{ts}* contains two point mutations which change a leucine residue (L) to an isoleucine (I) at position 616 and a serine residue (S) to a leucine (L) at position 779 in HAT7 and HAT10, respectively, as indicated. Allele *prp1Δ227-249* contains a deletion in the N-terminus as indicated. Numbers relate to amino acid positions of Prp1 as listed in the database (Gene Bank [http://www.ncbi.nlm.nih.gov] accession no.: Q12381). **B** At the restrictive temperature (35 °C) the pre-mRNA of *tbp1* containing three introns accumulates as shown in a Northern analysis. After three hours at 35 °C no spliced mRNA is detectable anymore. The complete *tbp1* gene was used as a probe. This gene encodes the TATA-binding protein (acc. no.: CAA17067).

Prp31 is also a *bona fide* highly conserved splicing factor. Interestingly, specific mutations in human PRP31 are associated with autosomal dominant retinitis pigmentosa (Vithana *et al*. 2001). In *S. pombe* a temperature sensitive allele *prp31-E1^{ts}* was identified in a screen

designed to isolate mutants defective in pre-meiotic S-phase. This mutant turned out to be temperature sensitive for vegetative growth and accumulated pre-mRNA at the restrictive temperature of 35 °C (Bishop *et al*. 2000).

Epistasis mapping by crossing a strain containing the allele *prp31-E1*^{ts} with a strain containing *prp1-4*^{ts} or *prp1-127*^{ts} (Figure 3.1 A), respectively, followed by tetrad analysis shows that *prp31-E1*^{ts} is synthetically lethal with *prp1-4*^{ts}, but not with *prp1-127*^{ts}. That is, cells with the allele *prp1-4*^{ts} in the same genetic background with *prp31-E1*^{ts} cannot grow even at the permissive temperature of 25 °C, whereas cells containing the ts alleles, *prp31-E1*^{ts} and *prp1-127*^{ts} grow well at the permissive temperature (Figure 3.2 A, B). Sequence analysis of the mutant allele revealed that *prp31-E1*^{ts} contains two mutations changing a glutamic acid residue (E) to a lysine (L) at positions 133 and 408, respectively (Figure 3.2 C).

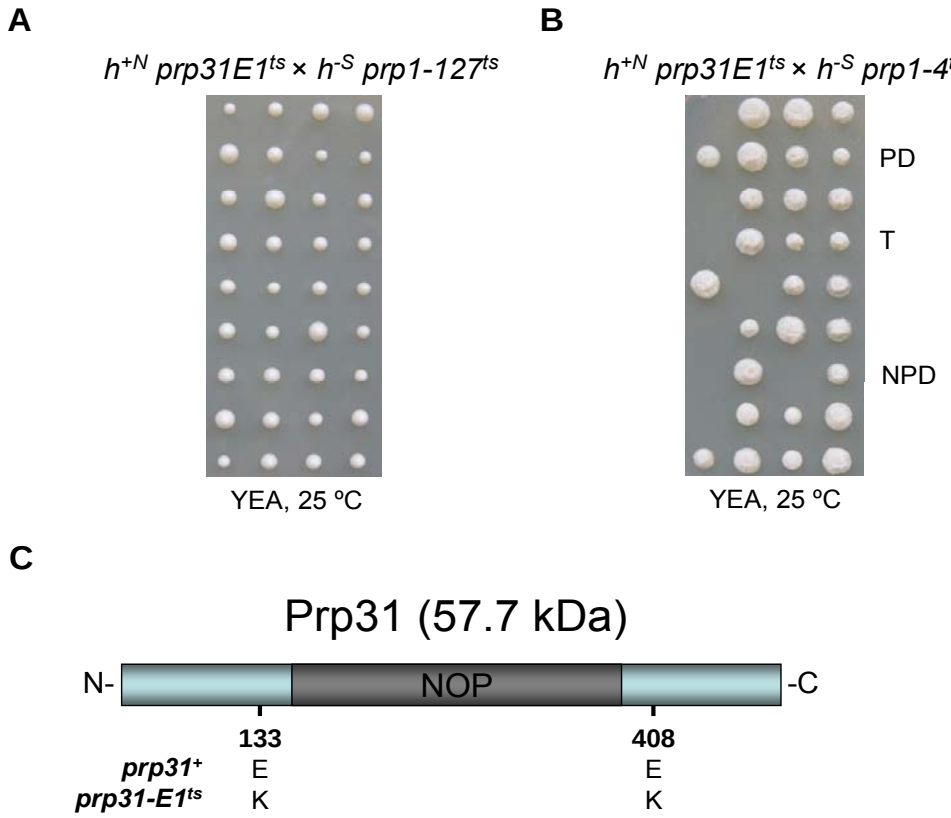

Figure 3.2 **A** and **B** *prp31-E1*^{ts} is synthetically lethal with *prp1-4*^{ts} but not with *prp1-127*^{ts}. Strains of opposite mating type were crossed as indicated. Tetrads were dissected and spores were grown on complete medium (YEA) at 25ºC. Spores containing *prp31-E1*^{ts} and *prp1-4*^{ts} alleles in the same genetic background do not form colonies at 25 °C, whereas spores containing *prp31-E1*^{ts} and *prp1-127*^{ts} alleles in the same genetic background grow at 25 °C. Strains HE624, UR100 and SL168 were used for this analysis (Table 5.2). PD: parental ditype; NPD: nonparental ditype; T: tetratype. Number of tetrads dissected for each cross: 54. **C** Diagram of Prp31 showing a central NOP domain. Two pointmutations were identified in *prp31-E1*^{ts} which change a glutamic acid residue (E) to a lysine (L) at positions 133 and 408, respectively, as indicated.

3.3 Prp1 and Prp31 are found in pre-catalytic spliceosomes

The classical genetic analysis of these alleles suggest a specific functional relationship between Prp1 and Prp31 in which, particularly, the HAT-motifs of Prp1 play an important role. Indeed, it has been shown over the last 10 years, while isolating and characterizing spliceosomal sub-particles that Prp1 and Prp31 are found associated with pre-catalytic spliceosomal particles, but not with post-catalytic spliceosomal complexes (Carnahan *et al*. 2005; Ohi *et al*. 2007). Based on these observations, the research group of N. F. Käufer used the presence of Prp1 and Prp31 together with base paired U4/U6 snRNA as markers to define pre-catalytic spliceosomal particles found *in vivo* in fission yeast (Bottner *et al*. 2005).

3.4 Expressing a mutant Prp1 stalled spliceosomes before activation

Mutations in the N-terminus of Prp1 lead to the accumulation of pre-catalytic spliceosomes containing the five snRNAs and unspliced pre-mRNA. An extensive deletion-mutation analysis of the N-terminus of Prp1 revealed that structural integrity of the N-terminus is required for the activation of pre-catalytic spliceosomes containing the five snRNAs U1, U2, U5 and U4/U6, but not for the assembly of splicing competent spliceosomes (Lützelberger *et al*. 2010).

In the following the isolation and characterization of pre-catalytic spliceosomal particles from a strain expressing the N-terminal deletion mutation Prp1Δ227-249 will be described (Figure 3.1 A, Table 5.2). Briefly, this strain expresses a wild-type *prp1* gene fused to a repressible promoter called *nmt1-8*. This promoter can be repressed by the addition of thiamine (+Thi) to the culture medium. The gene expressing the N-terminal mutation was fused with a Myc-epitope tag and is expressed by the natural *prp1* promoter. When the medium does not contain thiamine (-Thi) both genes are expressed. Adding thiamine (+Thi) to the medium leads to the repression of the wild-type *prp1* gene, whereas Myc-Prp1Δ227-249 is still expressed. Under this condition, when solely Myc-Prp1Δ227-249 is expressed (+Thi) cells stop dividing (Lützelberger *et al*. 2010).

Pre-catalytic spliceosomes were purified by the tandem affinity purification (TAP) method using TAP-tagged Prp31 from growing (-Thi) and arrested (+Thi) cells (Materials and Methods). Material eluted from the first (T) and second affinity column (C), respectively, was separated by SDS-PAGE, transferred onto nitrocellulose membrane and probed with an anti-TAP antibody to detect Prp31. In both cases (-Thi and +Thi) signals corresponding to Prp31-CM (63kDa) were detected on the western blot (Figure 3.3 A, lanes 3 and 4 and lanes 7 and 8). This blot was also probed with an anti-Myc antibody (αMyc) to detect Myc-Prp1Δ227-249. Myc-Prp1Δ227-249 was found associated with Prp31 in arrested cells (+Thi; Figure 3.3 B, lanes 7 and 8), but not with Prp31 in growing cells (-Thi; Figure 3.3 B, lanes 3 and 4). Probing the material eluted from the columns on a Western blot with an antibody against Prp1 (αPrp1) revealed that Prp31 in growing cells (-Thi) is exclusively associated with wild-type $Prp1^{wt}$ (-Thi; Figure 3.3 C, lanes 3 and 4). This indicates that Prp1Δ227-249 associates only with Prp31 complexes, if no $Prp1^{wt}$ is available in the cell. In other words, the presence of a functional $Prp1^{wt}$ completely outcompetes the mutant protein. This phenomenon is called allelic exclusion. It is an indication that Prp1 plays a very sensitive role at this stage of the splicing process.

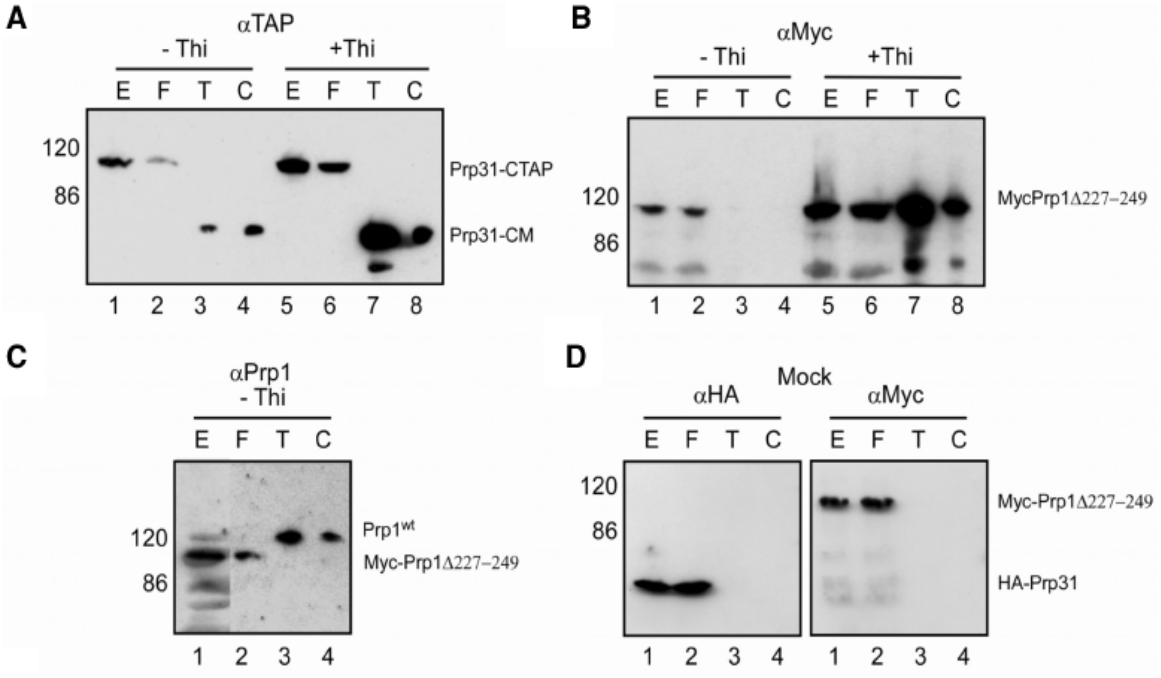

Figure 3.3 Purification of splicing complexes associated with Prp31. Cells expressing Myc-Prp1Δ227-249 and Prp31-CTAP were cultured in 5L of medium with (+Thi) and without (-Thi) and collected after 18 hours of growth. Cell extracts were used for tandem affinity purification (TAP) of splicing complexes. Protein extract (E, 0.02 % of total), flow through (F; material not binding to the first affinity column), eluate from the first affinity column (IgG-Sepharose after incubation with TEV protease; T, 5 % of the eluate) and eluate from the calmodulin resin after the second affinity step (C, 5 % of the eluate) was separated with SDS-PAGE, immunoblotted and probed with the indicated antibodies. **A** Blot probed with anti-TAP antibodies: αTAP to detect Prp31-CTAP and Prp31-CM. **B** Blot probed with anti-Myc antibodies: αMyc to detect Myc-Prp1Δ227–249. **C** Blot probed with anti-Prp1 antibodies: αPrp1 to detect $Prp1^{wt}$ (wild-type) and Myc-Prp1Δ227–249. **D** Mock, cell extracts were made from a strain expressing HA-Prp31 and Myc-Prp1Δ227–249 after 18 h in medium +Thi and applied consecutively to both columns as described above. Blot probed with anti-HA antibodies: αHA to detect HA-Prp31; and probed with anti-Myc antibodies: αMyc to detect Myc-Prp1Δ227-249.

To investigate at which stage the mutant Prp1Δ227-249 interferes with the splicing process we determined which of the five U snRNAs we find in the TAP-Prp31 complexes eluted from the second affinity column (C). For this purpose RNA was isolated from the material of growing (-Thi) and arrested cells (+Thi). The RNA was used for RT-PCR to probe for the five snRNAs U1, U2, U4, U5 and U6. To establish the optimal conditions for the amplification of all five U snRNAs in this assay, we used total RNA isolated from whole cell extract (Figure 3.4 C and Materials and Methods). The results of the RT-PCR experiments show that both the TAP-Prp31 complexes isolated from growing (-Thi) and from arrested (+Thi) cells contain the five snRNAs U1, U2, U4, U5 and U6. However, the

TAP-Prp31 complexes which associate with Myc-Prp1Δ227-249 in arrested (+Thi) cells contain significantly more snRNA U1 than the TAP-Prp31 complexes associated with $Prp1^{wt}$ in growing (-Thi) cells (Figure 3.4 A B, lanes 1, arrows).

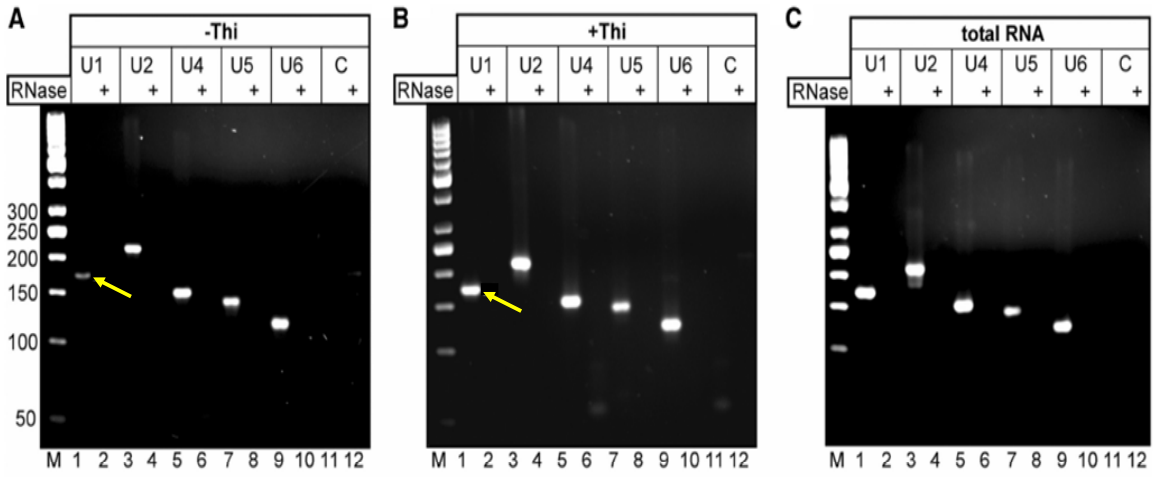

Figure 3.4 Analysis of the U snRNAs composition of splicing complexes associated with Prp31. **A** and **B** Material (+/-Thi) eluted from the last affinity column was analyzed for the presence of the U snRNAs. The RNA was isolated and used as template in RT-PCR. Relative levels of the five U snRNAs were determined using specific primers pairs amplifying U1, U2, U4, U5 and U6 snRNA as indicated on the top of each panel. The RT-PCR product of snRNA U1 is marked with a yellow arrow (Lane 1). **C** Total RNA prepared from a wild-type strain L972 was used as a positive control. All RNA samples were treated with Dnase I to remove possible DNA contaminants prior to reverse transcription. Complete removal of contaminating DNA was verified by RNase A treatment of the RNA as indicated (RNase+). RT–PCR products were separated in 3 % agarose gels and stained with ethidiumbromide. Lanes marked with 'C': negative control without template using the U1 primer pair. Lanes M: marker (50-bp ladder); numbers to the left indicate fragment length in base pairs.

Based on the results presented above, it is conceivable that the sole expression of Prp1Δ227-249 leads to the accumulation of spliceosomal particles containing the five U snRNAs. As discussed in Bottner *et al*. (2005) the sedimentation profile of Prp31 of growing cells revealed that the protein sediments in a range between 30-60S. The spliceosomal complexes sedimenting in this range appear to be a mixture of two pre-catalytic spliceosomal particles. One consists of U2, U5, U4/U6 (tetra-snRNP), whereas the other contains the five U-snPNPs including U1 (penta-snRNP). Therefore, the sedimentation profile of (TAP) purified Prp31 spliceosomal particles from arrested cells solely expressing Myc-Prp1Δ227-249 (+Thi) were compared with the Prp31 sedimentation profile of whole cell extract (WCE) from growing (-Thi) and arrested (+Thi) cultures.

Whole cell extract and the TAP-Prp31 purified material was size fractionated on a 10-30 % glycerol gradient. The gradient fractions were separated by SDS-PAGE, immunoblotted and probed with the appropriate Prp31 antibodies. The gradient profiles of Prp31 show that the TAP-Prp31 purified spliceosomes of the arrested cells sediment in the same range of 30-60S as the pre-catalytic spliceosomal complexes of growing cells (Figure 3.5).

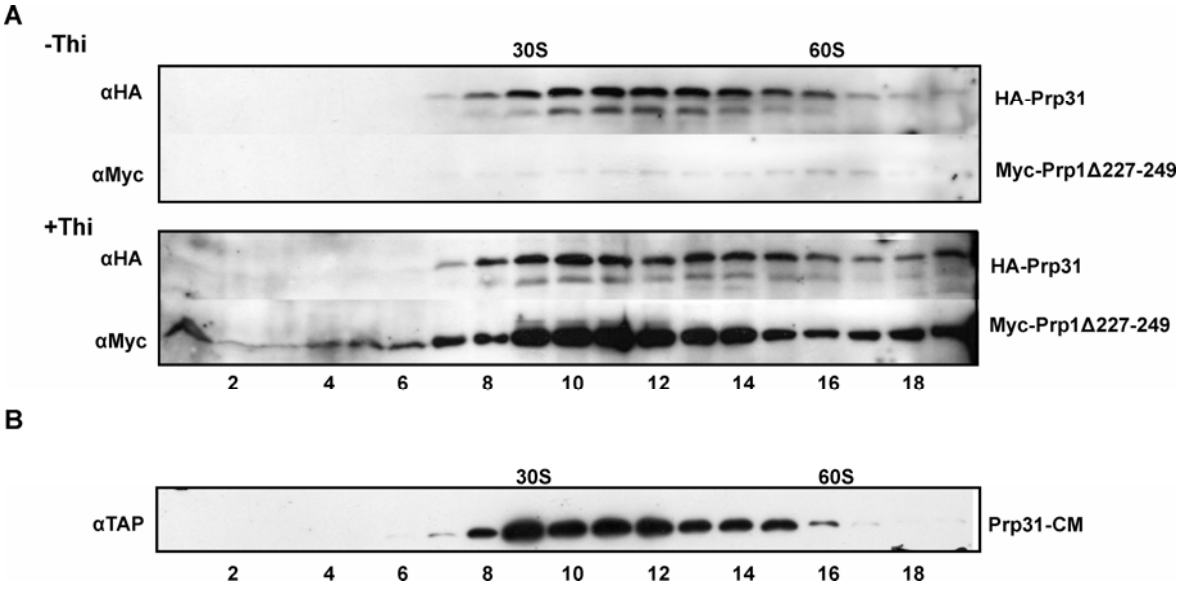

Figure 3.5 Distribution of Prp31 and Prp1Δ227–249 in large complexes. **A** Native extracts of the strain expressing HA-Prp31 and the N-terminal deletion Myc-Prp1Δ227–249 (497, Table 5.2) were prepared and separated on a 10–30 % glycerol gradient (top fraction 1 to bottom fraction 19). Fractions 1–19 were separated by SDS-PAGE, immunoblotted and probed as indicated with anti-HA antibodies (αHA) and anti-Myc antibodies (αMyc) to determine in the gradient the distribution of HA-Prp31 and Myc-Prp1Δ227–249, respectively. Panels –Thi: extract was isolated after 18 h in medium without thiamine; Panels +Thi: extract was isolated after 18 h in medium with thiamine. **B** Distribution of Prp31-CM. The eluate from the first affinity column (T) from extract after 18 h in medium +Thi was separated on a 10–30 % glycerol gradient (top fraction 1 to bottom fraction 19). Fractions 1–19 were separated by SDS-PAGE, immunoblotted and probed as indicated with anti-TAP antibodies (αTAP) to determine the distribution of Prp31-CM. The gradient was calibrated with small (30S) ribosomal subunits of *Escherichia coli* and large (60S) ribosomal subunit of *S.pombe*.

If the TAP-Prp31 particles contain pre-catalytic spliceosomes which have been stalled before activation, one might expect to find unspliced (pre-) mRNA in these particles. Therefore, the RNA isolated from TAP-Prp31 complexes from growing (-Thi) and arrested (+Thi) cultures was probed using RT-PCR for the presence of unspliced mRNA of the ribosomal protein genes *rpl29* and *rps27*. The gene *rpl29* contains a single intron of 53 nt

in size, whereas the gene *rps27* contains two introns of 38 and 206 nt in size, respectively. In growing cells *rpl29* and *rps27* are highly expressed and efficiently spliced as indicated by the RT-PCR analysis of total RNA. No unspliced mRNA was amplified for *rpl29* and *rps27*, respectively (Figure 3.6, lanes 5 and 11). However, the TAP-Prp31 complexes associated with Prp1Δ227-249 of arrested cells (+Thi) contain significant amounts of unspliced pre-mRNAs and only trace amounts of mature mRNA (Figure 3.6, lanes 3 and 9). Finally, in TAP-Prp31 complexes of growing cells (-Thi) neither pre-mRNAs nor mRNAs were found in significant amounts (Figure 3.6, lanes 1 and 7). These results are consistent with the notion, that the sole expression of Prp1Δ227-249 leads to spliceosomes containing the five snRNAs and unspliced pre-mRNA representing pre-catalytic spliceosomes, stalled before activation. It was also shown that these particles still contain basepaired U4/U6 (Lützelberger *et al*. 2010).

Taken together, the results presented above provide evidence that Prp1 plays an essential role in the activation of spliceosomes *in vivo*. Therefore, it was interesting to screen for more interaction partners of Prp1 with the expectation to find components which help to elucidate the specific function of Prp1 in the activation process.

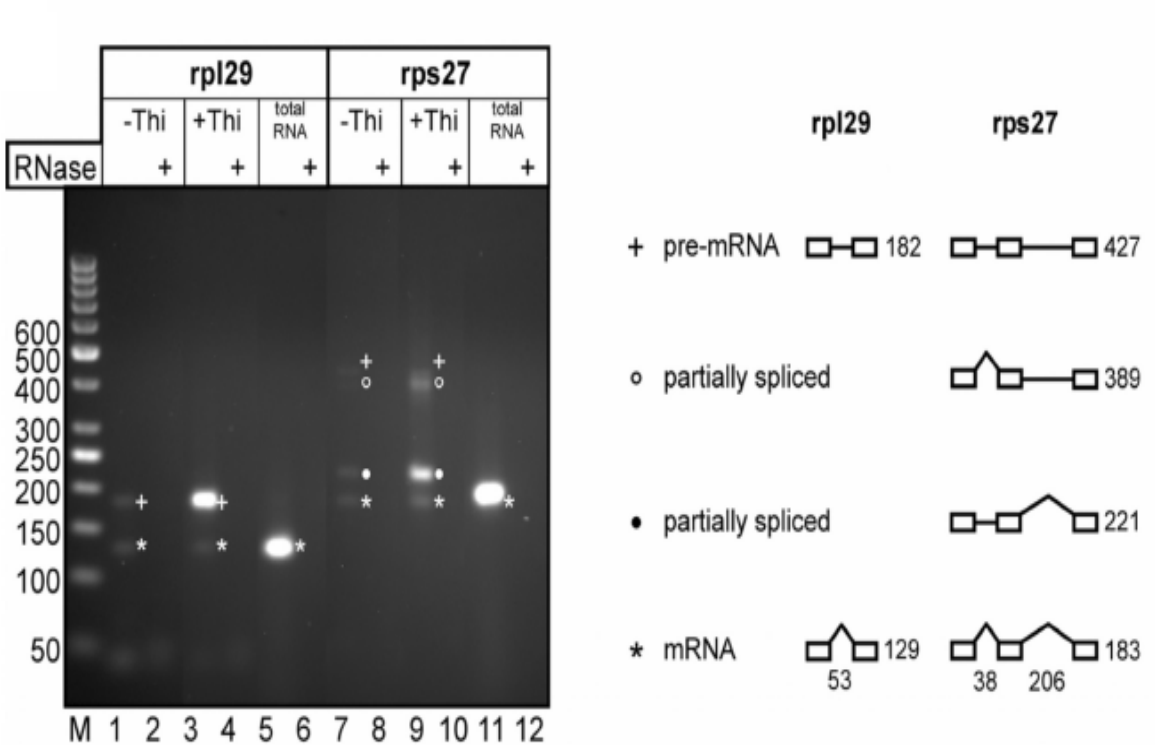

Figure 3.6 The RNA samples from **A** and **B** (Figure 3.4) were used to probe for the presence of pre-mRNA/mRNA of ribosomal genes *rpl29* and *rps27*. Primer pairs complementary to *rpl29* and *rps27* were used in RT-PCR. The exon/intron structure of both genes is shown to the right. Numbers below indicate intron length in nucleotides for both pre-mRNAs. Numbers to the right indicate expected RT-PCR product sizes in base pairs. All RNA samples were treated with Dnase I to remove possible DNA contaminants prior to reverse transcription. Complete removal of contaminating DNA was verified by RNase A treatment of the RNA as indicated (RNase+). RT–PCR products were separated in 3 % agarose gels and stained with ethidiumbromide. Lanes marked with 'C': negative control without template using the U1 primer pair. Lanes M: marker (50-bp ladder); numbers to the left indicate fragment length in base pairs.

3.5 Isolation of strains carrying extragenic suppressors of the *prp1-127^{ts}* allele

The strain HE624 containing the temperature sensitive (ts) allele *prp1-127^{ts}* was used to search for suppressors of *prp1-127^{ts}*. This ts allele carries a mutation changing glycine (G) to aspartic acid (D) at position 705 in HAT 8 (Figure 3.1 A). A shift of the cells carrying the *prp1-127^{ts}* allele to the restrictive temperature (35 °C) causes growth arrest which correlates with the accumulation of unspliced pre-mRNA of intron containing genes (Figure 3.1 B).

The ts strain was mutagenized with UV light and screened for growing colonies at 35 °C. Growing colonies were further characterized to determine the nature of the reversion. Either the mutant allele is reverted back to wild-type or contains additional mutations. The latter is known as an intragenic suppressor. If the mutation which enabled the cells to grow

at the restrictive temperature occurred in another gene, it is called an extragenic suppressor. Screening for extragenic suppressors of *prp1-127^{ts}* aims to identify interaction partners of Prp1

In order to identify strains carrying extragenic suppressors of the *prp1-127^{ts}* allele the colonies growing at 35 °C were crossed with the wild-type (wt) strain L975 containing *prp1^{+}*. Tetrad analysis was performed. Spores were grown at the permissive temperature (25 °C), subsequently replica plated and incubated at 35 °C. In case of intragenic suppression one would expect that all four spores of one ascus grow at 35 °C. In case of extragenic suppressors, one would expect three classes of tetrads (PD, parental ditype, NPD, non parental ditype, T, tetratype) at 35 °C, indicating independent assortment of a gene suppressing *prp1-127^{ts}*. The suppressor strains isolated with the above described procedure were then further genetically characterized to determine whether the suppressor genes are allelic or whether different loci are involved in the suppression of *prp1-127^{ts}*. These analyses revealed five different loci called *spp101-1* (suppressor of *prp1*), *spp102-1, spp104-1, spp105-1, spp106-1,* and *spp107-1* (Table 5.2).

3.6 Are the identified suppressors dominant or recessive?

The approach to isolate the suppressor genes by transforming a genomic library of the particular suppressor strain into the ts strain containing *prp1-127^{ts}* and then screening for growing colonies at the restrictive temperature (35 °C) requires to know, whether the suppressors are dominant or recessive in suppressing *prp1-127^{ts}*. Since fission yeast is a haploid organism a method developed by Prof. Gutz and others was used to select for diploid mitotically dividing cells (Materials and Methods; Gutz *et al*.1974). With this procedure diploids were produced at the permissive temperature (25 °C). These diploids were homozygous for the *prp1-127^{ts}/prp1-27^{ts}* alleles and heterozygous for the suppressor alleles *spp1/spp1^{+}* (Figure 3.7). The diploid strains grown at 25 °C were replated and tested for growth at the restrictive temperature. It was found that the suppressor alleles *spp101-1*, *spp102-1* and *spp107-1* are dominant, whereas *spp104-1*, *spp105-1* and *spp106-1* are recessive (Figure 3.7).

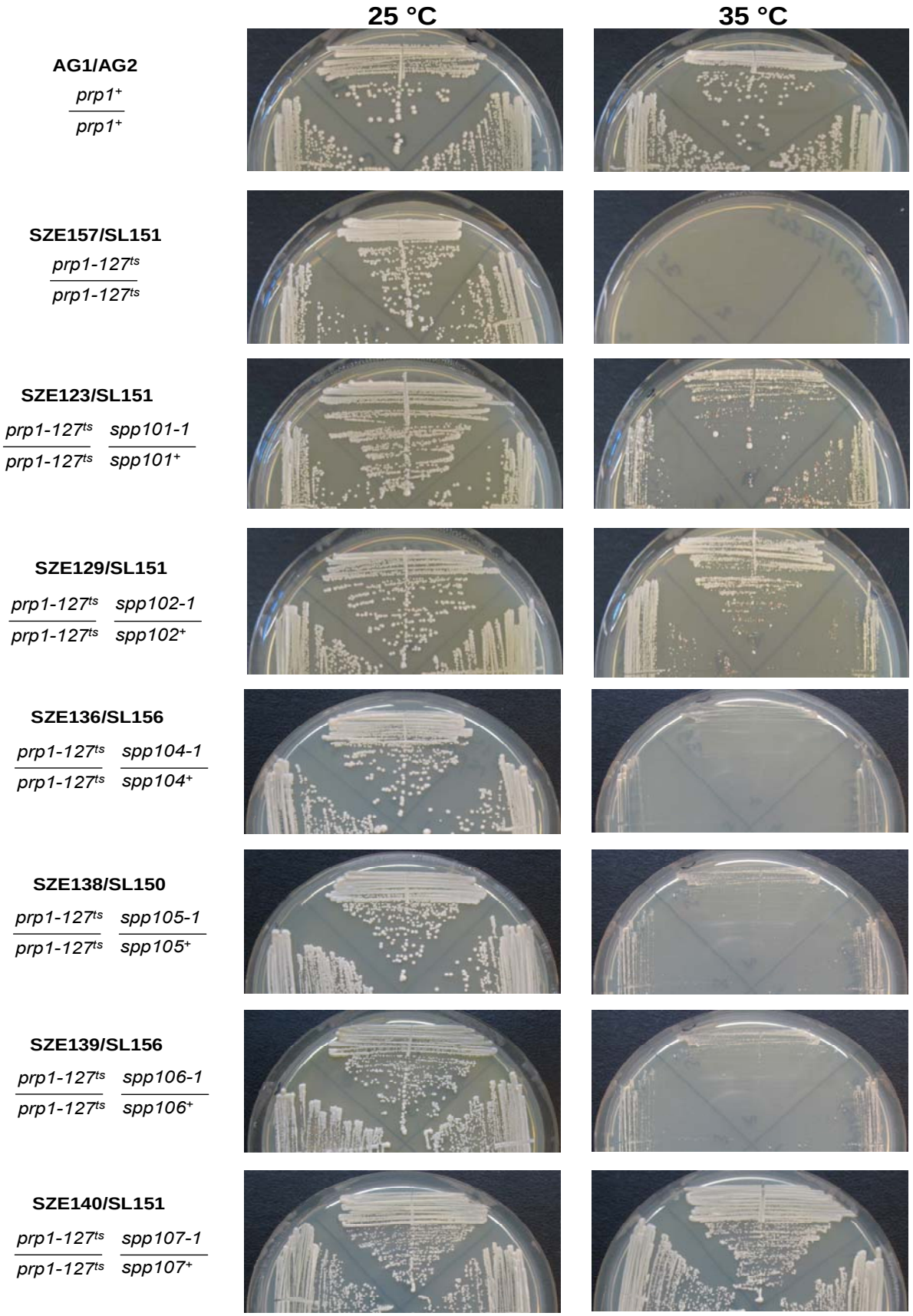
25 °C
35 °C
AG1/AG2
prp1+ / prp1+
SZE157/SL151
prp1-127ts / prp1-127ts
SZE123/SL151
prp1-127ts spp101-1 / prp1-127ts spp101+
SZE129/SL151
prp1-127ts spp102-1 / prp1-127ts spp102+
SZE136/SL156
prp1-127ts spp104-1 / prp1-127ts spp104+
SZE138/SL150
prp1-127ts spp105-1 / prp1-127ts spp105+
SZE139/SL156
prp1-127ts spp106-1 / prp1-127ts spp106+
SZE140/SL151
prp1-127ts spp107-1 / prp1-127ts spp107+

Figure 3.7 Determination of dominant and recessive suppressor alleles of *prp1-127^{ts}*. Diploid strains were constructed as described in the text. Strains used for the construction of diploids and their allelic state for *prp1* and the suppressor alleles are shown on the left of each panel. For the complete genotype see Table 5.2. After the construction of diploids at 25 °C the cells were plated on YEA medium incubated for six days at 25 °C and 35 °C as indicated above the panels. A diploid strain homozygous for *prp1-127^{ts}/prp1-127^{ts}* allele was used as a negative control and a diploid strain homozygous for *prp1^{+}/prp1^{+}* was used as a positive control.

3.7 Isolation of extragenic suppressors of the *prp1-127^{ts}* allele

In order to isolate the suppressor allele *spp102-1* a genomic library was constructed using the plasmid pAL19 and chromosomal DNA isolated from strain K29 *(spp102-1 prp1-127^{ts};* Table 5.2). pAL19 replicates as high copy number plasmid in *S.pombe* (Materials and Methods). The library was transformed into strain HE624 *(spp102^{+} prp1-127^{ts}*) and it was screened for colonies growing at the restrictive temperature (35 °C; Materials and Methods). The plasmids isolated from growing colonies containing the chromosomal DNA fragments of the suppressor strain K29 are called pAL19-K29. The additional number refers to the colony used to isolate the plasmid. From six growing colonies plasmids were isolated and the restriction enzyme patterns were analysed after cutting the plasmid DNA with Hin*dIII*/Eco*RI*. This analysis revealed that four of the plasmids show the same restriction pattern and carry a chromosomal DNA insert of about 11 kb in size (Figure 3.8 A and 3.8 B). The plasmid containing this insert from colony 1 was named pAL19-K291. To test whether pAL19-K291 can rescue the growth defect of the strain HE624, the isolated plasmid was transformed in HE624 and tested for growth at 35 °C. It was found that pAL19-K291 enabled the cells to grow at 35 °C (Figure 3.8 C).

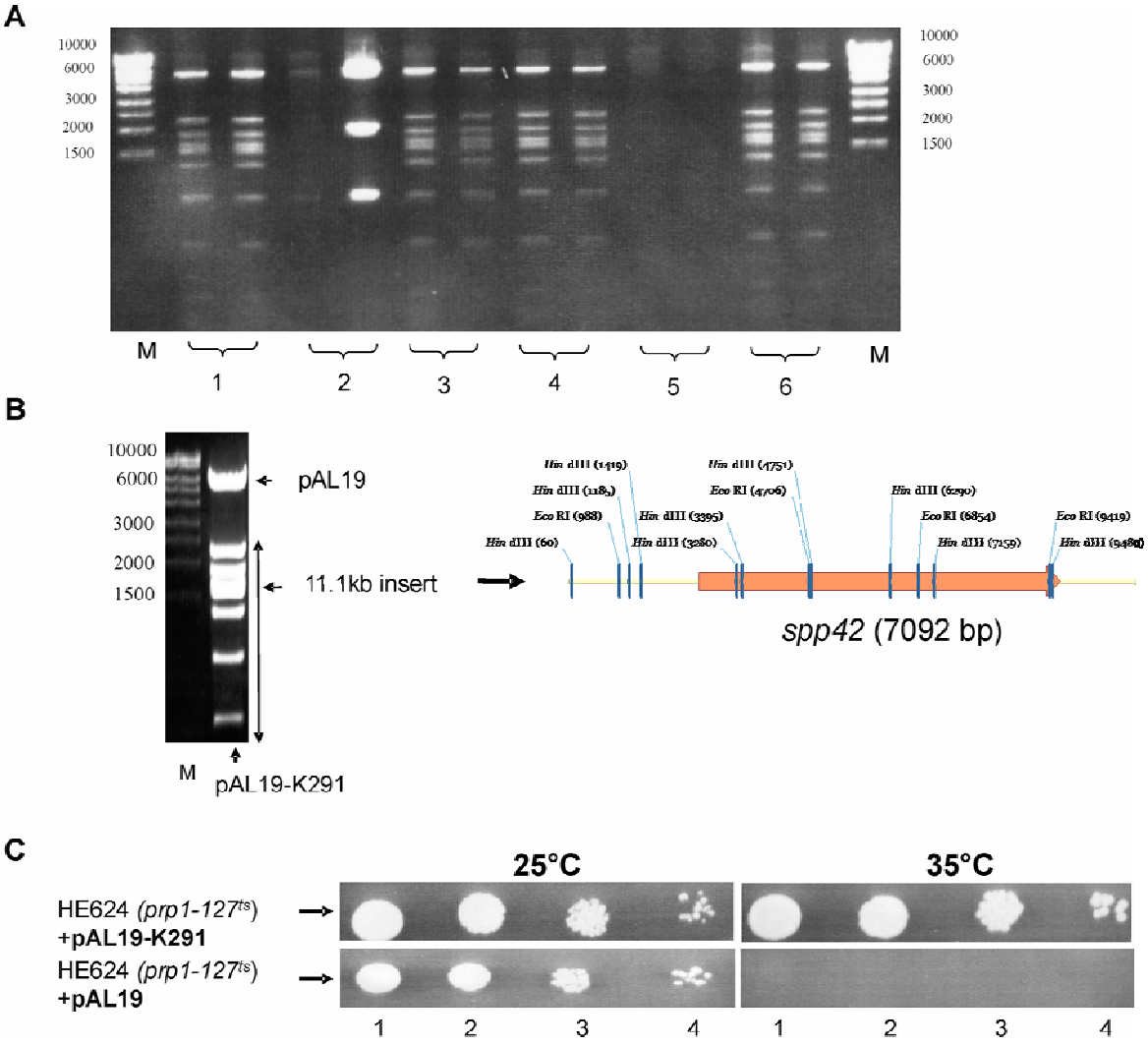

Figure 3.8 Analysis of plasmids suppressing the growth defect of strain HE624 at the restrictive temperature 35 °C. A genomic library of strain K29 containing the *spp102-1* allele was used in the screen. **A** Restriction analysis using *Hind*III and *Eco*RI in the assay. Brackets 1, 2, 3, 4, 5, 6 show the restriction of two plasmids isolated from six different colonies, respectively. The restricted plasmid DNA was separated in a 1 % agarose gel. **B** *Hind*III/*Eco*RI restriction digest with a plasmid preparation of one of the plasmids of Bracket 1. Sequencing analysis of the plasmid containing an 11.1 kb insert revealed that it contains one ORF comprising 7092 bp of a gene annotated in GDB *S. pombe* as *spp42* (SPAC4F8.12c). The plasmid containing this insert is now called pAL19-K291. Numbers in brackets indicate a position of restriction sites. Lane M: DNA fragments (High range DNA ladder) were used as size markers; numbers indicate fragment length in base pairs. **C** After (re)-transformation of plasmid pAL19-K291 into HE624 at 25 °C the transformed strain was spotted in a serial dilution series on the appropriate medium (Materials and Methods) and incubated at 25 ° and 35 °C as indicated. A pAL19 plasmid without insertion was used as control. 10^4, 10^3, 10^2, 10 cells were spotted at positions 1, 2, 3, 4, respectively.

Further analysis revealed that the genomic insert of the plasmid contains only one open reading frame comprising 7092 bp which encodes a spliceosomal protein known as

Spp42/Prp8 (Figure 3.9, GDB: SPAC4F8.12c). The insert of pAL19 can either be a true extragenic suppressor or a high copy suppressor of *prp1-127^{ts}*, because the plasmid pAL19 is propagated at a high copy number per cell.

To investigate whether pAL19-K291 harbors a true extragenic or a high copy suppressor, the plasmid was used as a template for PCR and three overlapping fragments spanning the complete *spp42* ORF (Figure 3.9 B) were produced, transformed into the strain HE624 and screened for growth at 35 °C. If one of these fragments contains a suppressing mutation, it can convert the wild-type allele of *spp42* in the genome by gene conversion and the growth defect of this strain containing the *prp1-127^{ts}* allele will be rescued. Indeed, a significant amount of growing colonies at 35 °C were found only when the PCR fragment containing the first 2500 bp of *spp42*, was used for transformation (Figure 3.9 B, fragment 1, results not shown). These results indicate that this fragment carries a mutation. Sequencing analysis revealed the suppressing mutation at position 253 downstream of the ATG start codon of *spp42.* At this position in fragment 1 (Figure 3.9 B) guanine (G) was replaced by adenine (A) which at the amino acid level changes a lysine residue (K) to glutamic acid (E) at position 85 in the N-terminus of Spp42/Prp8 (Figure 3.9 C). Thus, *spp102-1* is a true extragenic suppressor of the *prp1-127^{ts}* allele.

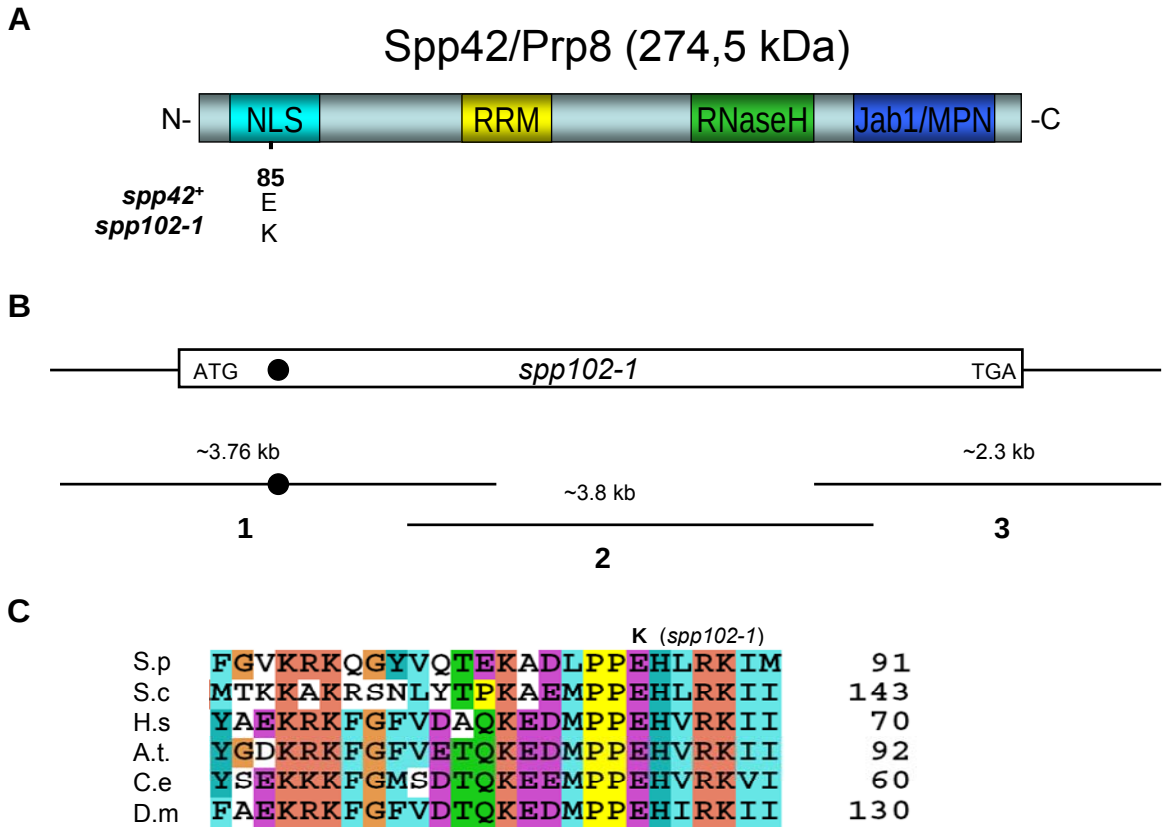

Figure 3.9 A Diagram of Spp42 showing known motifs recognized throughout the protein. The pointmutation found in *spp102-1* which suppresses *prp1-127^{ts}* changes a glutamic acid residue (E) to a lysine (K) at amino acid position 85 in the very N-terminus of Spp42 as indicated. NLS, RRM, RNaseH and Jab1/MPN domains are shown (explanation see text). **B** Three overlapping DNA fragments spanning *spp42* (1,2,3 as indicated) were produced by PCR. Plasmid pAL19-K291 containing the *spp102-1* suppressor was used as a template. A black circle denotes a mutation changing guanine to adenine at position 253 downstream of the start codon (ATG). Numbers above each PCR fragment indicate approximate DNA sizes in kb. Stop codon (TGA) is indicated. Transformation of fragment 1 into the strain HE624 can rescue its growth defect at 35 ºC. **C** Alignment of the suppressor mutation site in Spp42 with sequences from other organisms. Clustal X alignment of the *S.pombe* (S.p) Spp42 protein (Gene Bank acc. no.: CAB11062) with sequences from *S. cerevisiae* (S.c; CAA80854); *Homo sapiens* (H.s; AAC61776); *A. thaliana* (A.t; AAD55467); *C. elegans* (C.e; AAA27977); *D. melanogaster* (D.m; AAF58573). The numbers on the right indicate the position of the amino acids of the protein sequence. A lysine residue (K) (indicated on top of the alignment) is found instead of a glutamic acid residue (E**)** at the mutation site. Spp42 contains 2363 aa. The orthologue of Spp42 in *S. cerevisiae* is called Sc Prp8 and in *H. sapiens* Hs Prp8 or U5-220K.

Stimulated by the success of the approach described above, libraries of chromosomal DNA from strains K7 (*spp101-1 prp1-127^{ts}*) and K116 (*spp107-1 prp1-127^{ts}*, Table 5.2) were constructed and transformed into the strain HE624 containing the *prp1-127^{ts}* allele. Then, it was screened for colonies growing at the restrictive temperature (35 °C). From growing

colonies plasmids were isolated and analysed by restriction analysis as described above. Plasmids containing chromosomal DNA from the suppressor strain K7 are called pAL19-K7. Plasmids containing chromosomal DNA from strain K116 are called pAL19-K116. The restriction analyses of the plasmids isolated from both screens revealed that the colonies selected carry plasmids harboring genomic DNA fragments of different size (Figure 3.10 A and Figure 3.11 A). Further analysis showed that pAL19-K71, pAL19-K72, pAL19-K73 and pAL19-K1161 contain the same DNA region of chromosome 3. When these plasmids were retransformed into the strain HE624 all four perfectly rescued the growth defect at 35 °C as shown here for pAL19-K71 and pAL19-K1161 (Figure 3.10 B). All four chromosomal inserts have at least one complete ORF in common encoding a protein known as Ptc1 belonging to the family of 2C protein phosphatases (GDB: SPCC4F11.02). In addition, sequencing these inserts also revealed that the plasmids most likely contain the wild-type allele of *ptc1*, suggesting that it might be a high copy number suppressor of *prp1-127^{ts}*. To test this assumption, PCR was used to amplify *ptc1* from a wild-type (wt) strain (L972), as well as from the strain K7 containing the suppressor *spp101-1*. The PCR products were inserted into the vector pAL19 resulting in plasmids pAL19-wt-ptc1 and pAL19-K7-ptc1 (Materials and Methods). These plasmids were also transformed into the strain HE624 and then tested for growth at 25 °C and 35 ºC. Both plasmids were able to rescue the growth defect caused by *prp1-127^{ts}* at 35 °C (Figure 3.10 C). These results confirm that all *ptc1* genes isolated are wild-type alleles and act as high copy number suppressor of *prp1-127^{ts}* .

Furthermore, while screening the genomic libraries from strains K7 and K116 we also isolated pAL19-K74 and pAL19-K1162. These plasmids also rescue the growth defect of strain HE624 at 35 °C after retransformation (Figure 3.10 B) Sequence analysis revealed that the plasmids carry a wild-type allele of *mas5* (GDB: SPBC1734.11). These results suggest that *mas5* is also a high copy suppressor of *prp1-127^{ts}*. The ORF of the annotated gene *mas5* predicts a protein belonging to the family of Hsp40 proteins.

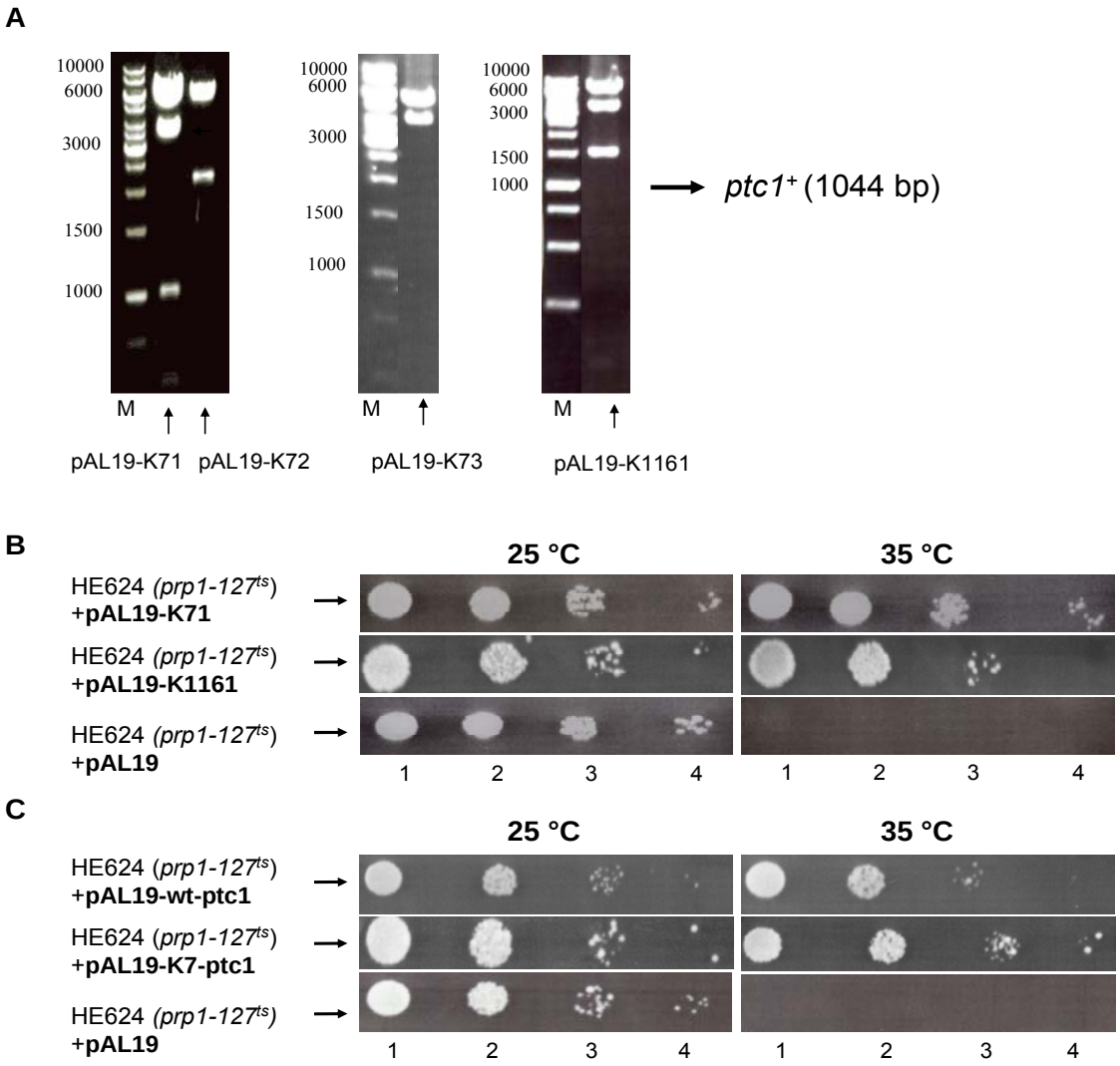

Figure 3.10 Analysis of plasmids suppressing the growth defect of strain HE624 at the restrictive temperature of 35 °C. **A** Restriction analysis using *Hind*III and *Eco*RI in the assay. pAL19-K71, pAL19-K72, pAL19-K73 plasmids were isolated after transforming the genomic library of strain K7 (*spp101-1 prp1-127^{ts}*) into strain HE624 and screening for growth at 35 °C. The same approach was used to isolate pAL19-K1161 plasmid from the genomic library of strain K116 (*spp107-1 prp1-127^{ts}*). The restriction digest was separated in a 1 % agarose gel. Lane M: DNA fragments (1kb DNA ladder) were used as size markers; numbers indicate fragment length in base pairs. Sequence analysis of the plasmids indicated that they contain the same ORF comprising 1044 bp of a gene annotated in GDB (GeneDB) *S. pombe* as *ptc1* (SPCC4F11.02). **B** After transformation of the plasmids pAL19-K71 and pAL19-K1161 into HE624 at 25 °C the transformed strains were spotted in a serial dilution series on the appropriate medium (Materials and Methods) and incubated at 25 ° and 35 °C, as indicated. A pAL19 plasmid without insertion was used as a control. 10^4, 10^3, 10^2, 10 cells were spotted at positions 1, 2, 3, 4, respectively. **C** Plasmids pAL19-wt-ptc1 and pAL19-K7-ptc1 (Materials and Methods) containing *ptc1* from strains L972 and K7 (Table 5.2), respectively, were transformed into HE624 at 25 °C. The transformed strains were spotted in a serial dilution series on the appropriate medium (Materials and Methods) and incubated at 25 ° and 35 °C, as indicated. A pAL19 plasmid without insertion was used as control. 10^4, 10^3, 10^2, 10 cells were spotted at positions 1, 2, 3, 4, respectively.

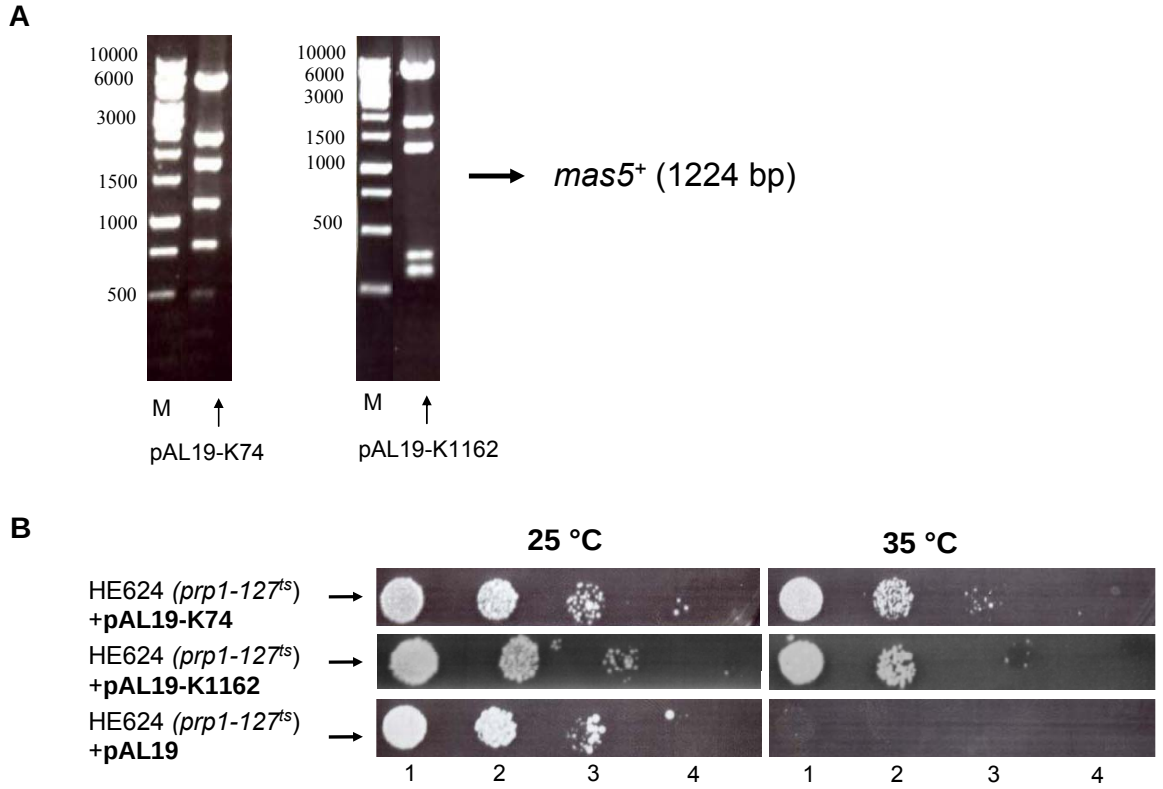

Figure 3.11 Analysis of plasmids suppressing the growth defect of strain HE624 at the restrictive temperature 35 °C. Genomic libraries of strain K7 (*spp101-1 prp1-127^{ts}*) and strain K116 (*spp107-1 prp1-127^{ts}*) were used in the screens. **A** Restriction analysis using *Hind*III and *Eco*RI in the assay. Plasmids pAL19-K74 and pAL19-K1162 were isolated from growing colonies of the screen described in the legend of Figure 3.10. The restriction digest was separated in a 1 % agarose gel. Lane M: DNA fragments (1kb DNA ladder) were used as size markers; numbers indicate fragment length in base pairs. Sequence analysis of the inserts indicated that they share one common ORF comprising 1224 bp of a gene annotated in GDB *S. pombe* as *mas5* (SPBC1734.11). **B** After transformation of the plasmids pAL19-K74 and pAL19-K1162 into HE624 at 25 °C the transformed strains were spotted in a serial dilution series on the appropriate medium (Materials and Methods) and incubated at 25 ° and 35 °C as indicated. A pAL19 plasmid without insertion was used as control. 10^4, 10^3, 10^2, 10 cells were spotted at positions 1, 2, 3, 4, respectively.

Collectively, screening for suppressors of *prp1-127^{ts}* with genomic libraries in high copy number plasmids led to the isolation of the suppressor allele *spp102-1*. This gene encodes a *bona fide* highly conserved spliceosomal component, called Spp42/Prp8, which is known as the central organizer of the spliceosome (Discussion). For *spp101-1* and *spp107-1* this approach was not successful. However, the screen revealed two high copy number suppressors of *prp1-127^{ts}*. Remarkably, both gene products, the 2C protein phosphatase and the Hsp40 family protein appear to function in the stress response pathway (Shiozaki *et al*. 1994).

3.8 Isolation of *spp101-1* using a candidate approach identifies the *lin1* gene as a true suppressor of *prp1-127^{ts}*

While genetically characterizing the suppressor strains it was observed that *spp101* is linked to the mating type locus *mat1* (GDB: SPBC23G7.09). The mating type locus (*mat1*) is located on the right arm of chromosome 2. The *prp1* gene is also located on chromosome 2 (Figure 3.12 A). Strains carrying the suppressor alleles *spp101-1* (K7), *spp101-2* (K54) and *spp101-3* (K104), respectively, were used and crossed with strain C1 (Table 5.2). The results of tetrad analyses performed by Karen Böhme (Diplomarbeit, Institut für Genetik, TU Braunschweig 2003) allowed us to determine the relative distance between the *spp101* and the *mat1* locus by calculating the recombination frequencies as suggested by Perkins (Perkins, 1949). This calculation revealed that the distance between *spp101* and *mat1* might be in the range of 50 cM. Since the calculation was done with low numbers of tetrads, it was only used for orientation on the chromosome (Materials and Methods). Based on these calculations the suppressor allele is expected to be located somewhere proximal to the centromere of chromosome 2 (Figure 3.12 A). Therefore, I "walked" *in silico* from the *mat1* locus towards and across the centromere. The criteria for becoming a candidate gene were:

a) an orthologue of the annotated *S.pombe* gene existed in other organisms and was characterized experimentally as a factor involved in spliceosomal splicing, or

b) the annotation of the gene sequence referred to the gene product as involved in RNA processing or interacting with RNA processing factors.

Within one Mb upstream of the *mat1* locus 12 genes have been found which met these criteria (Table 3.1).

Table 3.1 *spp101-1* suppressor candidates of *prp1-127^{ts}*

Name	kDa	Systematic name	Description	Position on ChrII	Colonies
mat1	4.8	SPAC823.15	Mating-type M-specific polypeptide Mi	2114219..2114347	
sab14	13.1	SPBC29A3.07c	U2 snRNP-associated protein Sf3b14 homolog (predicted)	2050258..2050695	113
lsm3	10.6	SPBC9B6.05c	U6 snRNP-associated protein Lsm3	1822126..1822673	232
cwc16	34.1	SPBC18H10.10c	complexed with Cdc5 protein	1788047..1789031	117
sme1	9.6	SPBC11G11.06c	Sm snRNP core protein	1764504..1765133	154
smd3	11.0	SPBC19C2.14	Sm snRNP core protein	1702182..1702628	128
prp38	24.4	SPBC19C2.08	U4/U6 x U5 tri-snRNP complex subunit Prp38 (predicted)	1690148..1690978	221
cdc28	121.2	SPBC19C2.01	DEAD/DEAH box ATP-dependent RNA helicase	1670582..1673749	92
pop3	35.1	SPBC21B10.05c	WD repeat protein. May play a role in mRNA maturation.	1662947..1663891	192
cwf7	21.3	SPBC28F2.04c	Involved in mRNA splicing.	1577407..1578009	215
lin1	47.3	SPBC83.09c	U5-snRNP associated GYF domain protein.	1525693..1526965	1580
prp5	52.4	SPBP22H7.07	WD repeat protein required for both cell cycle progression at G2/M and pre-mRNA splicing	1446112..1447533	179
msl1	12.6	SPBC8D2.09c	U2 snRNP-associated protein	1375313..1375721	107

The data bank information of this table was retrieved from: http://www.genedb.org/genedb/pombe/. Name: gene name; kDa: kilodalton; ChrII: chromosome II; Colonies: number of growing colonies at 35ºC after transformation of 1.6×10^8 cells of a strain containing the *prp1-127^{ts}* allele (HE624) with 1.5 µg PCR fragment comprising the complete coding sequence of each locus. DNA for PCR was isolated from the suppressor strain *spp101-1 prp1-127^{ts}* (K7).

Genomic DNA from the suppressor strain K7 (*spp101-1 prp1-127^{ts})* was isolated and PCR products comprising the complete ORFs of the candidate genes listed in Table 3.1 were produced. The PCR products were transformed into the strain HE624 (*spp101^{+} prp1-127^{ts})*. Then, it was screened for growth at the restrictive temperature (35 °C). The transformation of the 3 kb PCR fragment of the gene *lin1* (GDB: SPBC83.09c) into HE624 yielded 1580 growing colonies at 35 °C, whereas transformation of the other PCR fragments revealed never more than 232 colonies (Table 3.1). Subsequently the PCR fragment comprising *lin1* was subcloned into the plasmid pAL19, now called pAL19-K7-lin1. A sequence comparison between pAL19-K7-lin1 and a *lin1* allele isolated from strain HE624 by PCR and also subcloned in a pAL19 vector revealed one difference. At position 545 downstream of the ATG start codon either a Guanine or an Adenine was found revealing either the amino acid aspartic acid or asparagine at position 167 (Figure 3.12 B). The plasmid containing the PCR product from strain HE624 was called pAL19-wt-lin1.

Both plasmids pAL19-K7-lin1 and pAL19-wt-lin1 were transformed into the strain HE624 and tested for growth at 25 °C and 35 ºC. Plasmid pAL19-K7-lin1 indeed, but hardly, rescues the growth defect caused by *prp1-127^{ts}* at 35 °C (Figure 3.12 C). This high copy number effect might be the reason that the approach described before did not lead to the isolation of *spp101-1*. In addition, overexpression of the wt *lin1* allele on a high copy plasmid (pAL19-wt-lin1) does not rescue at all the growth defect at 35 °C (Figure 3.12 C). Thus, the candidate approach described here shows unambiguously that *spp101* is allelic with *lin1* (GDB: SPBC83.09c) and the mutated allele *lin1-1* from strain K7 is an extragenic suppressor of *prp1-127^{ts}*.

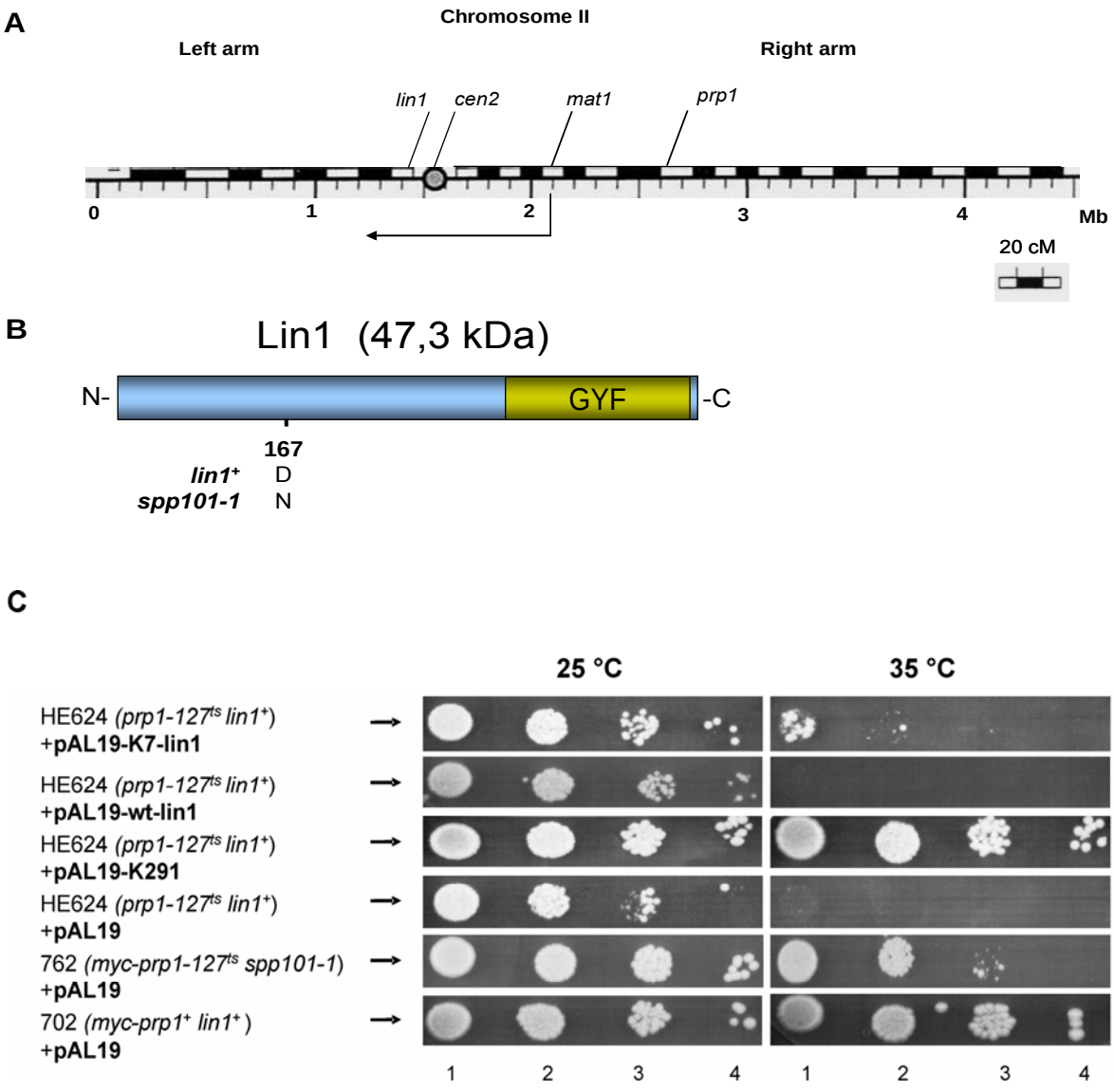

Figure 3.12 A Comparative map combining genetic and physical distances on chromosome II (from Egel R., "The Molecular Biology of *Schizosaccharomyces pombe*", 2004). Chromosome II has a short left and a long right arm; *cen2*, centromere region; *mat1,* mating type locus and *prp1*. The gene annotated under accession number SPBC83.09c was identified as the suppressor gene *spp101-1* suppressing *prp1-127^{ts}*. The gene at this locus is from now on called Sp *lin1* (see text for details). Numbers indicate length of the chromosome in Megabases (Mb). The arrow indicates the direction of *in silico* chromosome "walking". Each bar corresponds to 20cM. **B** Diagram of Lin1 showing a GYF domain at the C-terminus of the protein. One pointmutation was identified in *spp101-1* suppressing *prp1-127^{ts}*. This mutation changes as indicated an aspartate (D) to an asparagine (N) at position 167. **C** Plasmid pAL19-K7-lin1 and pAL19-wt-lin1 containing *lin1* from strain K7 and strain HE624, respectively, were transformed into HE624 at 25 °C. The transformed strains were spotted in a serial dilution series on the appropriate medium (Materials and Methods) and incubated at 25 ° and 35 ° C as indicated. HE624 carrying pAL19 plasmid without insertion was used as a negative control. HE624 carrying pAL19-K291 plasmid containing *spp102-1* suppressor, strain 762 (*myc-prp1-127^{ts} spp101-1*) and strain 702 (*myc-prp1 lin1^{+}*) were used as positive controls. For the complete genotypes see Table 5.2. 10^4, 10^3, 10^2, 10 cells were spotted at positions 1, 2, 3, 4, respectively.

The annotation of *lin1* in GDB shows an ORF encoding a protein of 47 kDa which display a GYF domain at the C-terminus (Figure 3.12). Interestingly, the alignment of the amino acid sequences from human (Hs), *S. cerevisiae* (Sc) and *S. pombe (*Sp) do not show a high degree of identical amino acids throughout the complete sequence (Table 3.2), however conserved signature sequences clearly define the GYF domain at the C- terminus. Conserved signature sequences also appear to define the N-terminal region (Figure 3.13). In mammalian cells the protein has been identified as U5-52K protein which is associated with the snRNP U5 and which also interacts with the cytoplasmic portion of the human T-cell receptor protein CD2 (Laggerbauer *et al*. 2005; Nielsen, 2007).

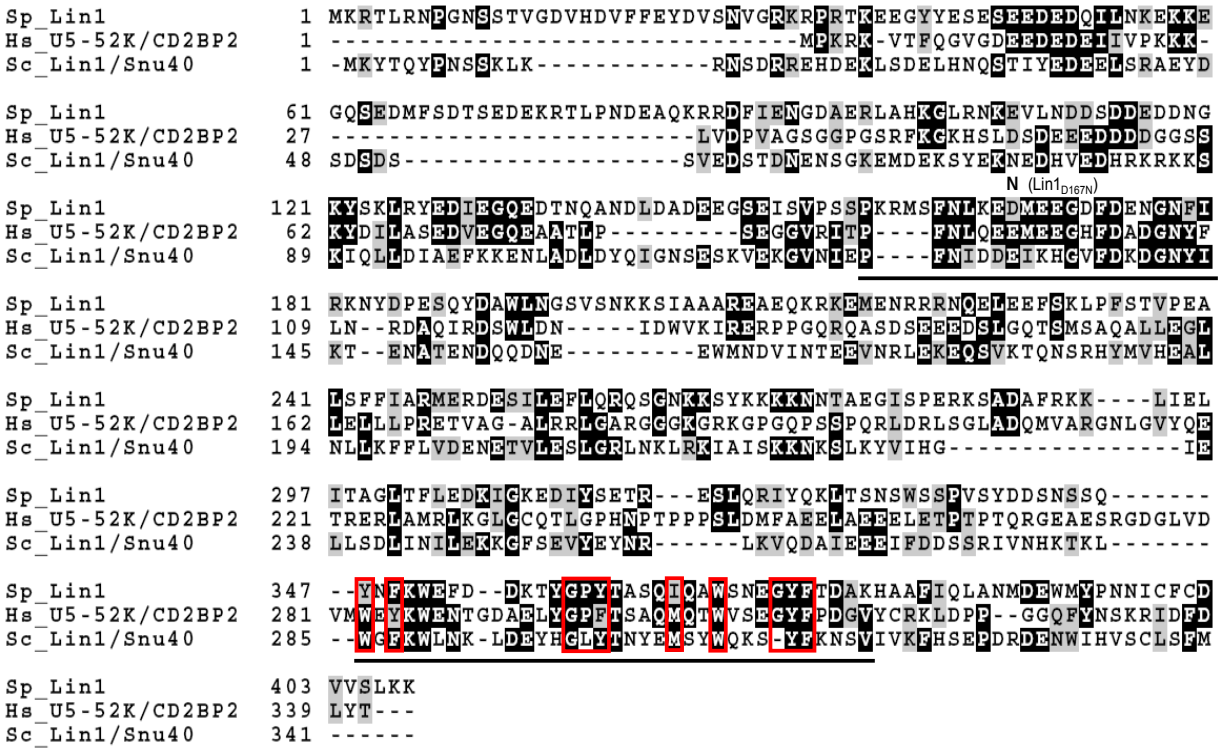

Figure 3.13 Clustal X alignment of the *S.pombe* (Sp) SPBC83.09c Sp_Lin1 (Gene Bank acc. no.: NP_595641) with its homologs from *H. sapiens* (Hs; acc. no.:NP_006101, called U5-52K/CD2BP2) and *S. cerevisiae* (Sc; acc. no.:NP_012026, Sc_Lin1/Snu40). Identical and similar residues are shaded in black and gray colour, respectively. An asparagine (N) (indicated on top of the alignment) is found instead of aspartate (D) at position 167 in the mutant protein $Lin1_{D167N}$. Conserved signature sequences are underlined. Residues that are characteristic of the GYF domain (W-x-Y-x_{6-10}-GPF-x_4-M-x_2-W-x_3-GYF (x represents nonconserved amino acid residue [Kofler M, Freund C, 2005]) are marked with red frames. The amino acid positions are indicated by numbers.

Table 3.2 Comparison of the ORF of Sp_ Lin1 with the Gene Bank

	Sp_Lin1 (*S.pombe*)	Sc_Lin1/Snu40 (*S.cerevisiae*)	U5-52K/CD2BP (human)
Length aa	408	340	341
% identity/similarity*	100/100	14.2/24.2	18.3/25.6

Length aa, number of amino acids found in the indicated amino acid sequences. Sp_Lin1, *S.pombe* (Gene Bank acc. no.: NP_595641), Sc_Lin1/Snu40, *S. cerevisiae* (acc. no.: NP_012026), U5-52K/CD2BP2, H. *sapiens* (acc. no.: NP_006101), * Percentage of overall amino acid identity/similarity with Sp_Lin1 ORF.

3.9 *lin1* is not essential for growth

In order to investigate whether *lin1* is essential for growth in *S. pombe*, a gene knock out cassette was constructed (see Materials and Methods). A DNA fragment comprising the 5'-flanking sequence of *lin1* followed by the *nat* gene, conferring nourseothricin resistance, and the 3'-flanking sequence of *lin1* was transformed into a diploid strain homozygous for $lin1^{+}$. On medium containing nourseothricin it was selected for growing cells and proper integration of the knock out cassette was determined by PCR (see Materials and Methods). Growing diploid colonies were used to induce sporulation followed by tetrad analysis (see Materials and Methods). If *lin1* is essential for growth one would expect to observe two viable spores growing to colonies in one ascus. If the gene is not essential for growth all four spores would grow to colonies. The results show that all four spores in a tetrad are viable at 30 ºC and grow to colonies indicating that *lin1* is not essential for growth (Figure 3.14). Intriguingly, this result indicates that a mutation in a non-essential gene can suppress the defect of an essential gene.

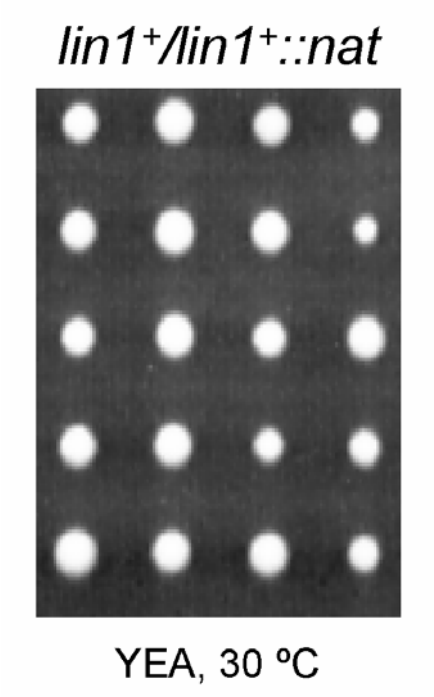

Figure 3.14 Lin1 is not essential for growth. Tetrad analysis of a diploid strain harboring one wild-type *lin1* allele and one allele replaced by nourseothricin resistance gene *nat* (*lin1*$^{+}$/ *lin1::nat*). The two spores containing the wild-type allele and the two spores containing the replaced allele grow up to colonies. Spores were grown on complete medium (YEA) for 5 days at 30 ºC.

3.10 In search of the suppressor function of Lin1

For further investigation, a *lin1* gene was constructed which was driven by the repressible *nmt1-8* promoter and the ORF was fused to the V5 epitope for immunodetection. This gene construct was integrated into the *leu1-32* locus on chromosome 2 and the wild-type *lin1*$^{+}$ gene was replaced by a nourseothricin resistance cassette via homologous recombination (see Materials and Methods). The *nmt1-8* promoter is repressible by adding thiamine (+Thi) to the culture medium. This strain is called 725. As shown in Figure 3.15 the cells expressing V5-Lin1 in medium without thiamine (-Thi) grow with the same growth rate as cells which do not express V5-Lin1 growing in medium with thiamine (+Thi; Figure 3.15 B). In addition, the strain 725 containing three different tagged proteins (V5-Lin1, Myc-Prp1, HA-Prp31) grows with the same growth rate as a wild-type strain (L975; Figure 3.15 A). This confirms that neither the absence of Lin1 nor the tagging of these three proteins lead to a measurable phenotype.

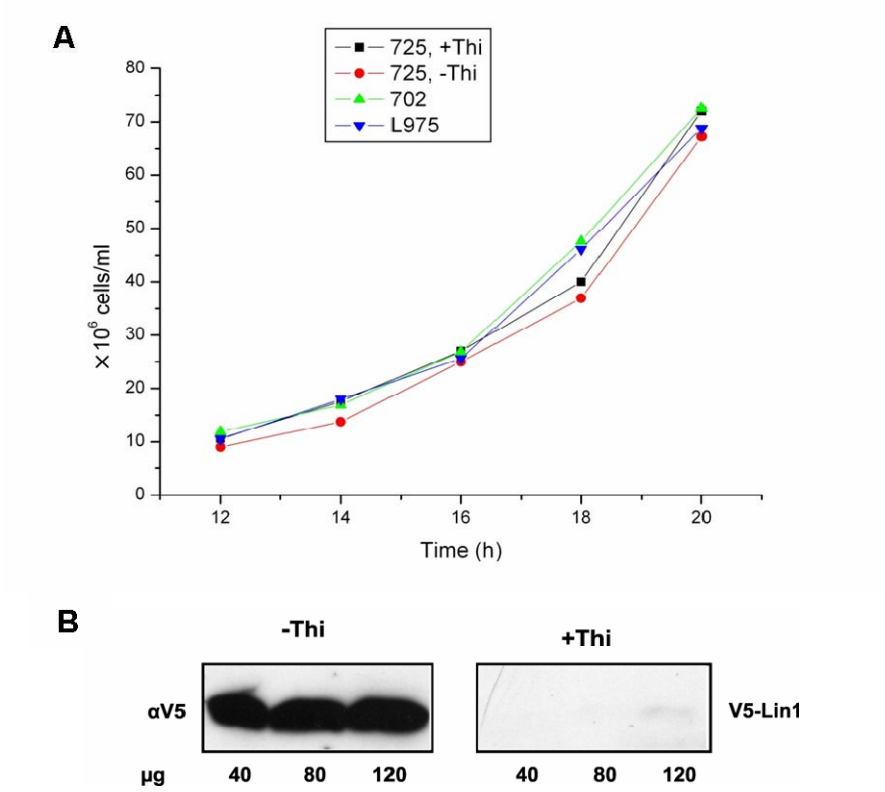

Figure 3.15 A Growth behaviour of strain 725 (*nmt1-8V5lin1*$^+$ *myc-prp1*$^+$ *HA-prp31*$^+$ *lin1*$^+$*::nat*) in medium with (+Thi; black squares) and without (-Thi; red cycles) thiamine. For comparison the growth curves of strain 702 (*myc-prp1*$^+$ *HA-prp31*$^+$) and a wild-type strain L975 (green triangles and blue flipped triangles, respectively) are presented. For the complete genotypes see Table 5.2. **B** Whole cell extract (WCE) prepared from strain 725 was separated by SDS-PAGE, immunoblotted and probed as indicated with anti-V5 antibodies (αV5) to detect V5-Lin1. 40, 80 and 120 µg of WCE were applied as indicated. Panel +Thi: WCE isolated after 19 h in medium with thiamine; Panel –Thi: WCE isolated after 19 h in medium without thiamine.

In a first attempt to explore whether Prp1 and Lin1 physically interact, whole cell extract (WCE) of cells grown without thiamine (-Thi) was immuno precipitated (IP) using anti-V5 (αV5) and anti-Myc (αMyc) antibodies to precipitate V5-Lin1 and Myc-Prp1, respectively. The immunoprecipitated material was separated by SDS-PAGE, transferred onto nitrocellulose and probed with αV5- and αMyc antibodies. The results show that under these conditions neither Lin1 co-precipitates with Prp1, nor Prp1 with Lin1 (Figure 3.16). This indicates that in growing cells no stable interaction between Prp1 and Lin1 is detectable.

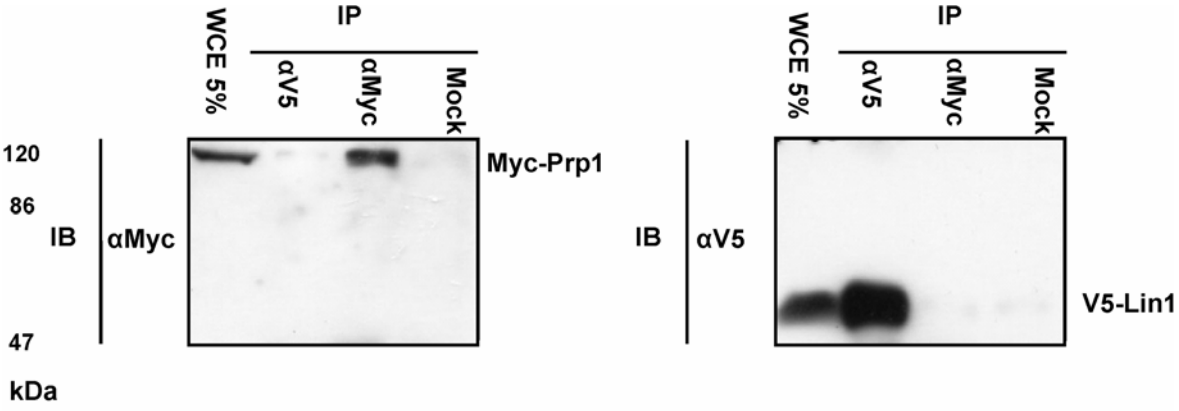

Figure 3.16 Lin1 does not stably interact with Prp1. Whole cell extract prepared from strain 725 (Table 5.2) grown for 19 hours in medium without thiamin (-Thi) was split and immunoprecipitated (IP) with anti-V5 (αV5) and anti-Myc (αMyc) antibodies as indicated. The immunoprecipitates were separated by SDS-PAGE, immunoblotted (IB) and probed as indicated with anti-V5 (αV5) and anti-Myc (αMyc) antibodies to detect V5-Lin1 and Myc-Prp1, respectively. Lane Mock: as a negative control the extract was incubated without addition of antibodies. Lane WCE 5 %: whole cell extract (5 % used for the immunoprecipitation) was used as a positive control. Numbers on the left indicate protein size in kilodalton (kDa).

Next, the sedimentation profile of pre-catalytic spliceosomal particles associated with Prp1 and Prp31 from cells expressing Lin1 (-Thi) were compared with the sedimentation profile of cells in which the expression of Lin1 was switched off (+Thi). For that purpose whole cell extract of cells grown with and without thiamine was size fractionated on a 10-30 % glycerol gradient. The gradient fractions were separated by SDS-PAGE, immunoblotted and probed for HA-Prp31, Myc-Prp1 and V5-Lin1 with the appropriate antibodies.

The sedimentation profile of Prp31 was used as a marker to determine the sedimentation range of steady state pre-catalytic spliceosomal particles *in vivo* as discussed before. There is no significant difference to detect between the sedimentation profiles of Prp31 in the presence (-Thi) and in the absence (+Thi) of Lin1. This means, the pre-catalytic spliceosomal particles sediment in the range of 30-60S (Figure 3.17 A, HA-Prp31 –Thi and +Thi). The same is true for Myc-Prp1. However, this statement requires some comment. During the course of this work the lysis procedure to prepare whole cell extract was optimized (Materials and Methods) revealing for Prp1 a more pronounced sedimentation profile showing a clear peak around 55S. However, contrary to the sedimentation profile of Prp31, Prp1 was also detected to sediment below 30S (Figure 3.17 A, Myc-Prp1 –Thi and +Thi). The sedimentation profile of V5-Lin1 shows a distinct particle sedimenting below 30S and peaking around 20S. (Figure 3.17 B, V5-Lin1). This

analysis indicates that in growing cells Lin1 is not stably associated with pre-catalytic spliceosomal particles which sediment in the range of 30-60 S.

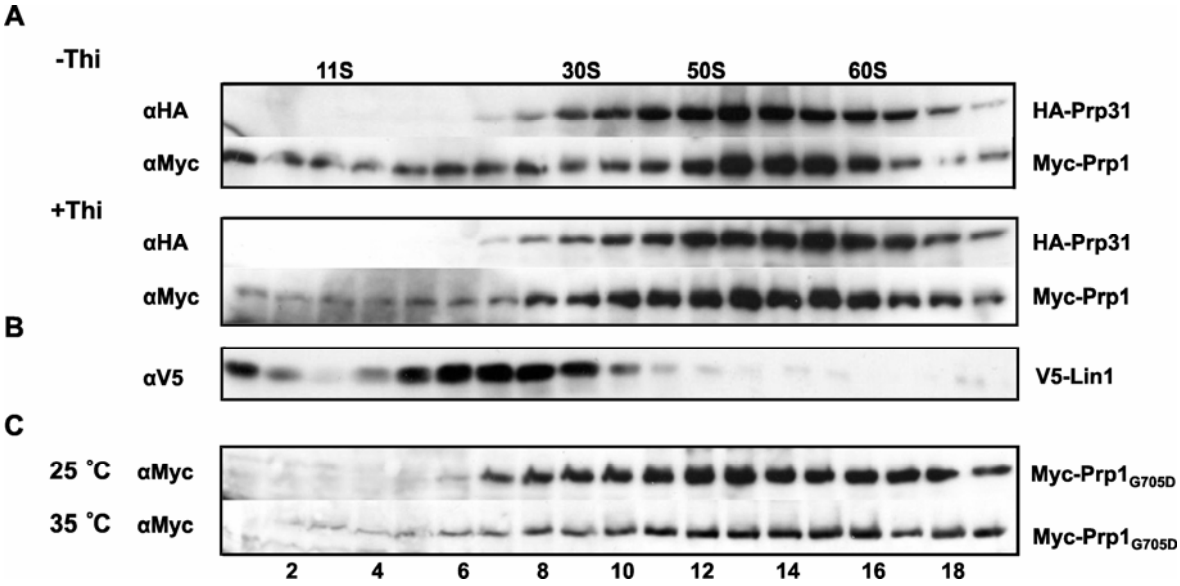

Figure 3.17 Distribution of Prp1, Prp1$_{G705D}$, Prp31 and Lin1 in large complexes. Whole cell extract (5 mg of total protein) was separated on a 10-30 % glycerol gradient (top fraction 1 to bottom fraction 19). Fractions 1–19 were separated by SDS-PAGE, immunoblotted and probed with appropriate antibodies. **A** Western blots were probed as indicated with anti-HA antibodies (αHA) and anti-Myc antibodies (αMyc) to determine the distribution of HA-Prp31 and Myc-Prp1, respectively. WCE was prepared from stain 725. Panels –Thi: WCE was isolated after 19 h in medium without thiamine; Panels +Thi: WCE was isolated after 19 h in medium with thiamine. **B** Western blot was probed as indicated with anti-V5 antibodies (αV5) to determine the distribution of V5-Lin1. WCE prepared from strain 725 grown for 19 h in medium without thiamine (-Thi) was used in this experiment. **C** Western blots were probed as indicated with anti-Myc antibodies (αMyc) to determine the distribution of Myc-Prp1$_{G705D}$. WCE was prepared from strain 748 (*myc-prp1-127^{ts}*). Panel 25 ºC: WCE was isolated from cells grown for 15 h in complete medium at 25 ºC; Panel 35 ºC: WCE was isolated from cells grown for 12 h in complete medium at 25 ºC and then shifted for 3 h to 35 ºC.

For further characterization of the 20S particle gradient fractions containing V5-Lin1 were pooled and immunoprecipitated using V5 antibodies (αV5). The immunoprecipitate (IP) was separated on SDS-PAGE and then probed in a Western analysis with anti-V5 antibodies demonstrating that αV5 immunoprecipitates V5-Lin1 from these fractions (Figure 3.18 A, arrow). In a second Western analysis the αV5 IP was probed with anti-Myc antibodies (αMyc) to check whether Myc-Prp1 co-precipitates with the V5-Lin1 20S particle. As shown in Figure 3.18 B, Prp1 appears not to co-precipitate with V5-Lin1. This

is consistent with the results shown in Figure 3.16 using whole cell extract for immunoprecipitation. These results indicate that the genetic interaction of the suppressor alleles is not based on a stable physical interaction between the two proteins.

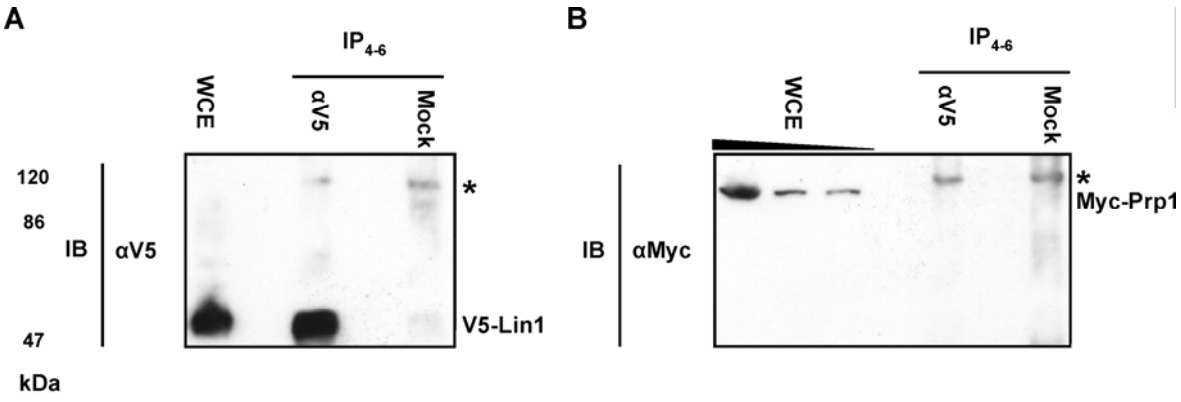

Figure 3.18 The 20S particle associated with Lin1 does not contain Prp1. Native extract from strain 725 after 19 hours in medium without (-Thi) thiamine was separated on a 10–30 % glycerol gradient (top fraction 1 to bottom fraction 19). The fractions 4-6 containing V5-Lin1 (Figure 3.17 B) were pooled and immunoprecipitated (IP_{4-6}) using anti-V5 antibodies (αV5) as indicated. The immunoprecipitate was separated by SDS-PAGE, immunoblotted (IB) and probed (**A**) with anti-V5 antibodies (αV5) and (**B**) with anti-Myc antibodies (αMyc) to detect V5-Lin1 and Myc-Prp1, respectively. Lane Mock: as a negative control material of fractions 4-6 was incubated without addition of antibodies. Lane WCE: whole cell extract from strain 725 was used as a positive control. Star indicates an unspecific signal. Numbers on the left indicate protein size in kilodalton (kDa).

Next, the V5-Lin1 immunoprecipitate (IP) was used to isolate RNA to investigate using RT-PCR analysis whether and which snRNA molecules are present in the 20S particle. Therefore, the RNA was probed for the five snRNAs U1, U2, U4, U5 and U6 as discussed above (page 19). The results of these RT-PCR experiments show that the V5-Lin1 20S complex contains U5 snRNA (Figure 3.19 A, B, C).

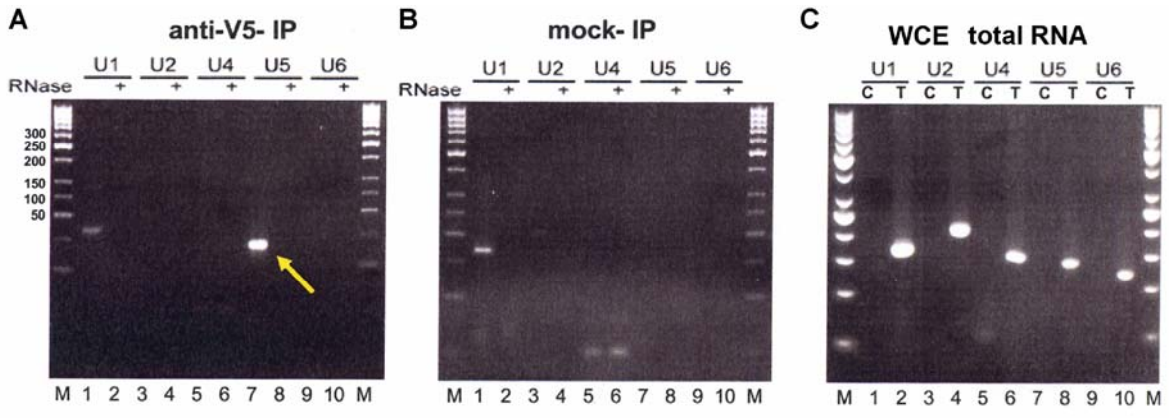

Figure 3.19 RT-PCR analysis to detect snRNA in the 20S Lin1 particle. **A** RNA isolated from the anti-V5 immunoprecipitate (αV5-IP_{4-6}, Figure 3.18) was analyzed by RT-PCR to determine the snRNA content. **B** RNA isolated from mock-IP_{4-6} (Figure 3.18). Specific primer pairs amplifying U1, U2, U4, U5 and U6 snRNA as indicated on the top of each panel were used in the analysis. The RT-PCR product of snRNA U5 is marked with a yellow arrow (Lane 7). **C** Total RNA prepared from a wild-type strain L972 was used as a positive control. Lane marked with 'T': total RNA. Lane marked with 'C': negative control without template. Lanes M: marker (50-bp ladder); numbers to the left indicate fragment length in base pairs. All RNA samples were treated with DNase I to remove possible DNA contaminants prior to reverse transcription. Complete removal of contaminating DNA was verified by RNase A treatment of the RNA as indicated (RNase+). RT–PCR products were separated in 3 % agarose gels and stained with ethidiumbromide.

Taken together, the results presented suggest that in growing cells Lin1 is found stably associated with a 20S particle containing the snRNA U5. This particle does not contain Prp1. In growing cells Prp1 is found associated with pre-catalytic spliceosomes sedimenting in the range of 30-60S, but clearly peaking at 55S. Lin1 is not associated with these pre-catalytic spliceosomal particles. In mammalian cells a 20S snRNP U5 particle containing snRNA U5 and the protein U5-52K (the Lin1 counterpart) has been characterized. This U5 snRNP particle in mammals also contains U5-102K which is the counterpart of Prp1 (Laggerbauer *et al*. 2005, Nielsen, 2007).

The experiments presented above revealed that *lin1-1* is a true extragenic suppressor of *prp1-127^{ts}* , however, it was observed that the growth behaviour of the suppressor strain at 35 °C differs from that of a wild-type strain. The suppressor strain grows slower than the wild-type at 35 °C (Figure 3.12 C, panel: 762 and panel: 702). Therefore, to quantitate the difference the doubling time (generation time) of the strains 702 (*prp1^{+} lin1^{+}*), 748 (*prp1-127^{ts} lin1^{+}*) and 762 (*prp1-127^{ts} lin1-1*) was determined at 25 and 35 °C (Table 5.2).
At 25 °C, which is the permissive temperature for the *prp1-127^{ts}* allele, the three strains reveal in complete medium a generation time of three hours (Figure 3.20 A). Shifting the ts

strain 748 from the permissive temperature (25 °C) to the restrictive temperature (35 °C) leads to a complete growth arrest after 3 hours. The suppressor strain (762), however, divides with a doubling time of six hours (Figure 3.20 B). For comparison, the wild-type strain (702) divides at this temperature with a doubling time of two hours (Figure 3.20 C).

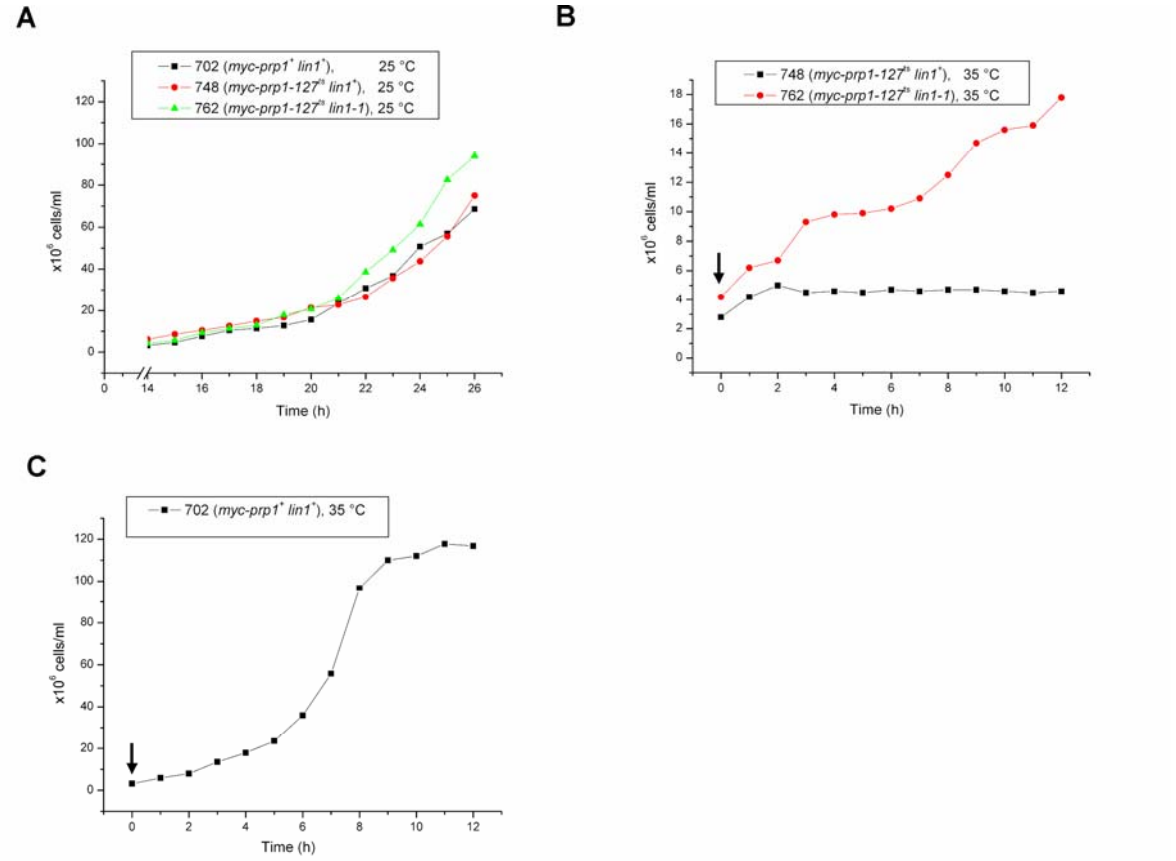

Figure 3.20 Growth behaviour of stains 702 (*myc-prp1*$^{+}$ *lin1*$^{+}$), 748 (*myc-prp1-127*^{ts} *lin1*$^{+}$), 762 (*myc-prp1-127*^{ts} *spp101-1/lin1-1*) at 25 ° and 35 ºC. (**A)** Strains 702, 748 and 762 (black squares, red cycles and green triangles, respectively) at 25 ºC. (**B)** Strains 748 and 762 (black squares and red cycles, respectively) and (**C**) strain 702 (black squares) were inoculated and grown for 14 hours at 25 ºC, then shifted to 35 ºC (arrow). The cells were grown in complete medium. For the full genotypes of the strains see Table 5.2.

The temperature sensitivity of the *prp1-127*^{ts} allele is due to one amino acid change in the eighth HAT motif (Figure 3.1 A). It has been shown that HAT motifs mediate protein to protein interactions, therefore the sedimentation profile of Prp1$_{G705D}$ was determined from cells growing at the permissive temperature (25 °C) and compared with the sedimentation profile of cells shifted for three hours to the restrictive temperature (35 °C) (Figure 3.20 B). As can be seen in Figure 3.17 C, the sedimentation profile of the mutant protein Prp1$_{G705D}$ does not significantly differ from the sedimentation profile of Prp1 suggesting

that the mutant protein still predominantly associates with pre-catalytic spliceosomes sedimenting in the range of 30-60S. However, one difference was readily observed. During the course of this work gradient analysis of whole cell extract (WCE) was standardized including the loading of 5 mg protein of WCE on each gradient. A comparison of the sedimentation profiles of the mutant protein $Prp1_{G705D}$ at the permissive (25 °C) and the restrictive temperature (35 °C) indicate that apparently lower amounts of $Prp1_{G705D}$ sediment in the range of 30-60S at the restrictive temperature of 35 °C (Figure 3.17 C). Based on this observation the steady state level of the wild-type $Prp1^{wt}$ and the mutant protein $Prp1_{G705D}$ at both the permissive (25 °C) and restrictive temperature (35 °C) was compared with each other. $Prp1^{wt}$ and the mutant protein $Prp1_{G705D}$ was visualized with the appropriate antibodies. For this purpose the strains 702, 748 and 762 were used in which the *prp1*-alleles were fused with the same Myc-epitope at the N-terminus of the protein. The *prp31* alleles were fused with an HA epitope at the N-terminus. The cyclin dependent kinase Cdc2 (Cdk1) was used as an internal control. It has been shown that the steady state level of Cdc2 does not significantly differ at the two temperatures. The results of these experiments show that shifting the wild-type strain for 3h to 35 °C leads to an increase of the steady state level of $Prp1^{wt}$ (Figure 3.21 panel: Prp1 prp^+ $lin1^+$). This increase is not due to a higher rate of transcription at this temperature, since transcriptome analysis data show that transcription of *prp1* is not increased in response to heat shock (Chen *et al*. 2003, GDB SPBC6B1.07). Therefore, the increase of Prp1 at 35 °C must be due to a post transcriptional mechanism. At the restrictive temperature (35 °C) the steady state level of the mutant protein $Prp1_{G705D}$ is visibly lower in the temperature sensitive strain when compared with $Prp1^{wt}$ in a wild-type strain (Figure 3.21 panels: $Prp1_{G705D}$ $prp1\text{-}127^{ts}$ $lin1^+$ and Prp1 prp^+ $lin1^+$ 35 °C). This indicates that $Prp1_{G705D}$ may be less stable than $Prp1^{wt}$ at 35 °C. In addition, the expression of $Lin1_{D167N}$ in the suppressor strain does not lead to the stabilization of $Prp1_{G705D}$ at 35 °C. In both cases the steady state level of $Prp1_{G705D}$ stays lower than the steady state level of $Prp1^{wt}$ at 35 °C (Figure 3.21 panel: $Prp1_{G705D}$ $prp1\text{-}127^{ts}$ *lin1-1*). These results indicate that the mechanism of suppression is not due to the stabilization of Prp1 by Lin1. Intriguingly, at the permissive temperature (25 °C) the steady state level of the mutant protein $Prp1_{G705D}$ is higher than the steady state level of $Prp1^{wt}$ (Figure 3.21 panels: $Prp1^{wt}$ $prp1^+$ $lin1^+$; $Prp1_{G705D}$ $prp1\text{-}127^{ts}$ $lin1^+$ and $Prp1_{G705D}$ $prp1\text{-}127^{ts}$ *lin1-1*). Both of these phenomena, the increase of the steady state level of $Prp1^{wt}$ at 35 °C as well as the increase of the steady state level of the mutant protein $Prp1_{G705D}$ at 25 °C

was unexpected. Particularly, the increase of the mutant protein $Prp1_{G705D}$ at 25 °C is surprising and I do not have an explanation for it yet.

The steady state level of Prp31 in the three strains at 25 and 35 °C were also compared. The spliceosomal protein Prp31 shows as $Prp1^{wt}$ an increased steady state level at 35 °C (Figure 3.21).

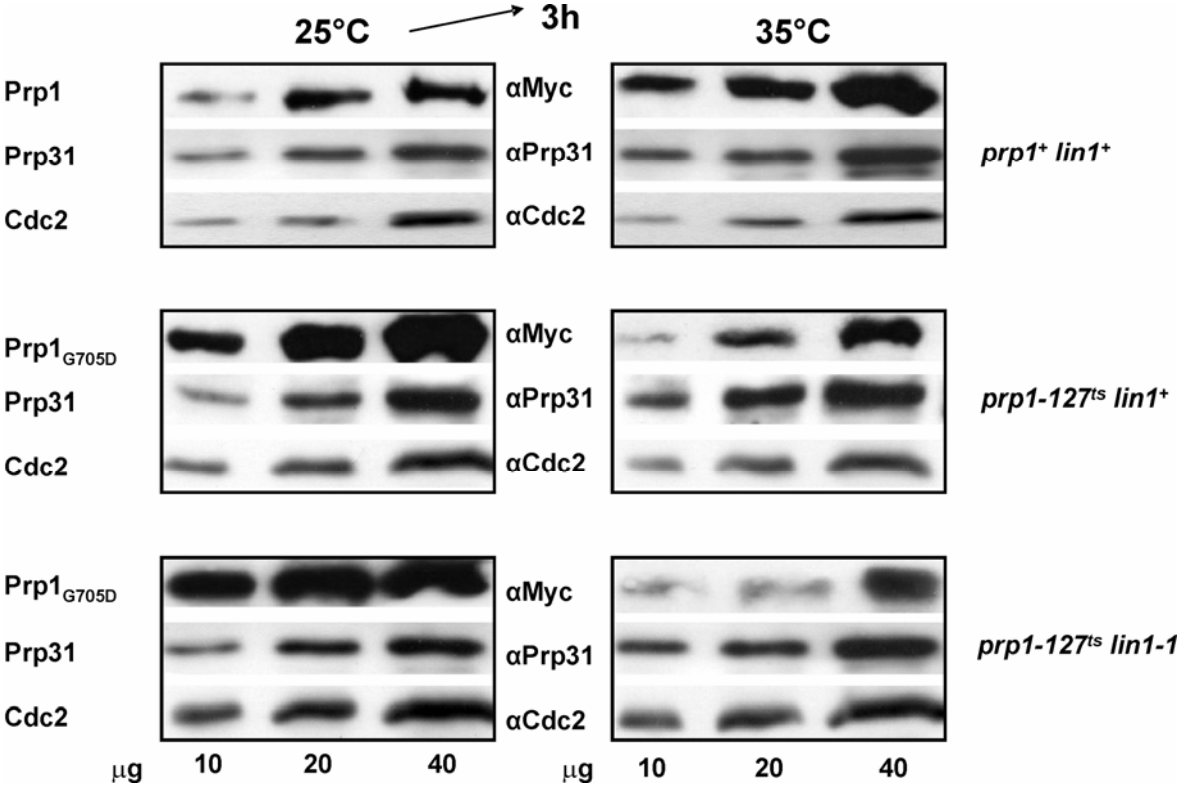

Figure 3.21 The mutation in HAT8 leads to destabilization of Prp1 at 35 ºC. Whole cell extract (WCE) from cells cultured 15 h at 25 ºC and then shifted for 3 h at 35 ºC was separated by SDS-PAGE, immunoblotted and probed with anti-Myc antibodies (αMyc), anti-Prp31 antibodies (αPrp31) and anti-Cdc2 antibodies (αCdc2) as indicated. αMyc visualizes Myc-Prp1 and Myc-$Prp1_{G705D}$, αPrp31 visualizes Prp31 and αCdc2 visualizes Cdc2. 10, 20, 40-µg of WCE were loaded as indicated. Panel *prp1*$^{+}$ *lin1*$^{+}$: WCE isolated from strain 702. Panel *prp1-127*^{ts} *lin1*$^{+}$: WCE isolated from strain 748. Panel: *prp1-127*^{ts} *lin1-1*: WCE isolated from strain 762. For the complete genotypes of the strains see Table 5.2.

Based on the results of these experiments and the fact that Prp31 exclusively binds to precatalytic spliceosomal particles sedimenting in the range of 30 to 60S (Figure 3.17) it is reasonable to suggest that in the temperature sensitive strain *prp1-127*^{ts} *lin1*$^{+}$ (748) at the restrictive temperature (35 °C) not all, but only parts of the pre-catalytic spliceosomal population is associated with the mutant protein $Prp1_{G705D}$. The mutant protein cannot mediate activation of pre-catalytic spliceosomes. Expression of the suppressor gene *lin1-1*, however, allows activation of those pre-catalytic spliceosomes associated with $Prp1_{G705D}$.

Therefore, it is conceivable that the mutation in the HAT8 domain impedes interaction with other components of the spliceosome needed for the initiation of a splicing event. $Lin1_{D167N}$ improves these interactions, thus operating as a chaperone. This interpretation is consistent with the observation that the suppression of *prp1-127^{ts}* by *lin1-1(spp101-1)* is by no means as good as the suppression of *prp1-127^{ts}* by *spp102-1* (Figure 3.12 C panel: HE624+pAL-K291 and panel: 762). The doubling time of the suppressor strain 762 (*prp1-127^{ts} lin1-1*) is three times longer and dividing cells show typical characteristics indicating extensions of cell cycle phases. Since 43 % of the genes in *S. pombe* including cell cycle regulatory genes contain introns, it is conceivable that it is the overall lower splicing activity which leads to this phenotype. The interpretation presented is consistent with the idea that in wild-type cells Lin1 might operate as effector molecule of Prp1. That is, the transient interaction of Lin1 with Prp1 during activation of pre-catalytic spliceosomes might improve the accessibility of the N-terminus of Prp1 which is phosphorylated by Prp4 kinase.

4 Discussion

In this study it was shown that the extragenic suppressor *spp101-1* of *prp1-127^{ts}* encodes Lin1 a 47 kDa protein displaying a GYF domain at the C-terminus. The orthologues in budding yeast and mammalia are called Lin1/Snu40 and U5-52K, respectively (Figure 3.13). Lin1 appears stably associated with a particle sedimenting at 20S containing snRNA U5, but not with pre-catalytic complexes sedimenting in a higher range. This 20S U5 particle does not contain Prp1 (Figure 3.18). In budding yeast and mammals Lin1/Snu40 and U5-52K, respectively, is also associated with the snRNP U5. The orthologue of Prp1, called Prp6, is not found associated with the U5 particle in budding yeast, but appears to be associated with U5 particles isolated from mammals (Stevens and Abelson, 1999; Stevens *et al*. 2001; Lagerbauer *et al*. 2005). Lin1 was not found stably associated with pre-catalytic higher order complexes (Figure 3.17), thus implying that the functional interaction suppressing the temperature sensitivity caused by *prp1-127^{ts}* is a transient interaction of the mutant proteins Prp1$_{G705D}$ and Lin1$_{D167N}$. The suppressing mutation in Lin1 was found in a conserved region of the N-terminus displaying a short E/D stretch. Interestingly, these short E/D stretches appear to be signatures of the Lin1 N-terminus, particularly conserved between the fission yeast and human protein (Figure 3.13). This observation suggests that the E/D stretches in the N-terminus of Lin1 might play a role in mediating the interaction between Lin1 and Prp1. This raises the question what is the function of the GYF domain in Lin1 during this interaction?

The few GYF domains which have been biochemically characterized recognize proline-rich sequences. The GYF domains which have been analysed recognized PPG as core motif, whereas the number of prolins (P) and the amino acids following the core motif can vary (Kofler and Freund 2005). These experiments have been conducted with the CD2 receptor binding protein (CD2BP2), which now in mammals turned out also to be involved in pre-mRNA splicing as U5-52K (Lin1/Snu40, Nielsen *et al*. 2007).

The second extragenic suppressor *spp102-1* of *prp1-127^{ts}* characterized in this work is Spp42/Prp8. This protein is the largest (2363 amino acids) and the most highly conserved spliceosomal protein which contains several different motifs including NLS (Nuclear Localisation Signal), RRM (RNA recognition motif), RNaseH fold and Jab1/MPN domain (Grainger and Beggs, 2005; Pena *et al*. 2008 and Figure 3.9). Spp42/Prp8 operates, as mentioned before, at the heart of the spliceosome providing a platform for regulatory proteins involved in activating a spliceosome (Newman and Nagai, 2010). The mutation in *spp102-1* suppressing *prp1-127^{ts}* is located in the very N-terminus changing a glutamic

acid (E) to lysine (K) at position 85. Based on sequence alignments this mutation is preceded by two prolines (P) and found in a highly conserved region, separating two clusters of positively charged residues which constitute a bipartite NLS (Nuclear Localization Signal [Grainger and Beggs, 2005; Boon *et al*. 2007]; Figure 3.9 and Figure 4.1). In addition, at amino acid position 5, 15 and 29 the core recognition motif PPG of GYF domains is found. The core recognition motif at position 15 and 29 is preceded by six and five consecutive prolines (P), respectively (Figure 4.1).

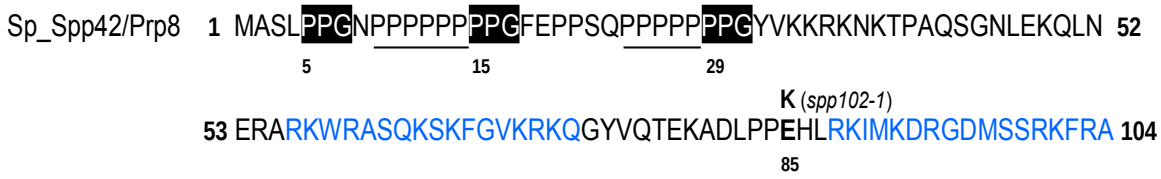

Figure 4.1 The N-terminal fragment of Spp42/Prp8 of *S.pombe* (Sp). PPG motifs are shaded black. Proline-rich sequences are underlined. Bipartite NLS sequences are in blue. A point mutation in the *spp102-1* suppressor leads to a lysine (K in bold type) instead of a glutamic acid residue (E in bold type) at position 85. The numbers indicate the position of the amino acids of the protein sequence. See the text for explanations.

The mutation in *spp102-1* at position 85 leads to very good suppression of *prp1-127^{ts}*, that is, normal growth behavior and wild-type morphology of the dividing cells at the restrictive temperature of 35 °C (Figure 3.12 C). However, when both supressor genes, *spp102-1* and *spp101-1* are expressed in cells containing *prp1-127^{ts}* the cells become temperature sensitive again, indicating a synthetic lethal interaction between *spp102-1* and *spp101-1* (results not shown). This genetic interaction is consistent with the idea that the GYF domain of Lin1 might interact with the proline-rich sequences in Spp42/Prp8. Indeed, in budding yeast a two hybrid screen using ScLin1/Snu40 as a bait revealed predominantely ScPrp8 as interaction partner, and it is claimed that ScPrp8 interacts with its proline-rich sequences in the very N-terminus with Lin1/Snu40 (Bialkowska and Kurlandzka, 2002).

Over the last years, we have identified multiple specific interactions between Prp1, Spp42/Prp8 and Prp4 kinase. The extragenic suppressor *spp42-1* of *prp4-73^{ts}* also encodes Spp42/Prp8. In this mutant allele at position 2248 an aspartic acid (D) is found instead of a glycine (G) in the wild-type. The mutant allele is synthetically lethal with *prp1-4^{ts}* (Bottner *et al*. 2005). As shown in this work, *prp1-4^{ts}* contains two mutations in HAT7 and HAT10, respectively, and is also synthetically lethal with *prp31-E1^{ts}* (Figure 3.2). The finding of these multiple genetic interactions is a clear hint that Prp1 (Prp6/U5-102K) is not just a

structural spliceosomal protein involved in the formation of a spliceosome as discussed in the literature (Makarov *et al*. 2000), but plays a regulatory role during formation and activation of spliceosomes.

We have demonstrated for the first time that Prp1 plays a regulatory role in the activation of spliceosomes. Deletions in the N-terminal domain lead to the accumulation of pre-catalytic spliceosomes containing the five snRNPs including base-paired U4/U6 and un-spliced pre-mRNA (Lützelberger *et al*. 2010). These stalled pre-catalytic spliceosomal complexes have been purified via tandem affinity purification using TAP-tagged Prp31 protein and were compared with affinity purified TAP-Prp31 complexes isolated from mitotically growing cells (Figure 3.3). As often it is the case, the discovery that Prp1 plays a regulatory role in the activation of spliceosomes was a somewhat fortunate coincidence. Since Prp1 is essential and it was attempted to investigate the consequences of the expression of mutations in *prp1*, the circumstance required the construction of cells in which two copies of *prp1* were expressed. Only one repressible promoter system was available. The wild-type gene was driven by the repressible promoter *nmt1*. That is, the wild-type gene could be repressed, leading to the sole expression of the mutant protein Prp1Δ227-249. However, in all cases the mutant protein was constitutively expressed leading under permissive growth conditions (without Thiamine, -Thi) to the co-expression of $Prp1^{wt}$ and mutant protein Prp1Δ227-249. The cells expressing $Prp1^{wt}$ and Prp1Δ227-249 show the same growth behaviour as cells expressing wild-type $Prp1^{wt}$ from two different gene copies (Lützelberger *et al*. 2010). Remarkably, under these for growth permissive conditions, hardly any mutant protein Prp1Δ227-249 associates with the spliceosomal particles sedimenting between 30 and 60S (Figure 3.5 A –Thi). Only wild-type $Prp1^{wt}$ associates with these pre-spliceosomal complexes (Figure 3.3 B and C, –Thi). That is, $Prp1^{wt}$ is dominant and completely outcompetes the mutant Prp1Δ227-249 when both proteins are co-expressed. This phenomenon is called allelic exclusion. The most famous classical example for allelic exclusion are the $lacI^{S}$ mutants described by Jacob and Monod (1961). By co-expression of $lacI^{S}$ repressor mutants, defect in binding Allo-Lactose or inducer IPTG, and $lacI^{+}$ in *Escherichia coli,* the *lac* operon remains repressed in the presence and absence of inducer. Here, the mutant molecule is dominant, however, it perfectly reflects the phenomenon of allelic exclusion in an haploid organism.

In *S. pombe* the sole expression of Prp1Δ227-249 leads to the accumulation of spliceosomal particles now exclusively associated with Prp1Δ227-249, sedimenting in the range of 30-60S and containing unspliced pre-mRNA (Figure 3.3 B, +Thi; Figure 3.6, +Thi

). This raises the question: what happens with Prp1Δ227-249 when $Prp1^{wt}$ is co-expressed? There are many hints that Prp1 molecules not associated with pre-catalytic spliceosomes become readily degraded to fragments of 85 and 47 kDa (results not shown). However, since co-expression of these two genes is in this case an artificial set up in the organism, this degradation process was not further investigated. Instead, more focus was given to the characterization of the stalled spliceosomal complexes when solely the mutant protein Prp1Δ227-249 was expressed. Indeed, ongoing research indicates that the stalled pre-catalytic spliceosomal complexes contain proteins of the Nineteencomplex (NTC), and it appears that Prp4 kinase is not stably associated to this stalled pre-catalytic complex (Lützelberger and Käufer, personal communication).

It has been shown previously that pre-catalytic spliceosomes containing U1, U2, U5 and U4/U6 exist *in vivo* independently of the presence of Prp1 (Bottner *et al*. 2005). The results presented in this work now provide evidence that the N-terminus of Prp1 is not necessary for the formation of a pre-catalytic spliceosome containing the five snRNAs, however, the structural integrity of the N-terminus of Prp1 is required that a pre-catalytic spliceosome can be activated (Lützelberger *et al*. 2010). Based on this observation and the fact that the deletion Prp1Δ227-249 contains phosphorylation sites of Prp4 kinase, we suggest that phosphorylation of Prp1 by Prp4 kinase is part of the process in which spliceosomes are activated. It is still unclear what the molecular consequences are, when Prp1 is phosphorylated by Prp4 kinase *in vivo*.

As discussed in the introduction, a few examples of regulated splicing are known. Several genes are regulated by pre-mRNA splicing during meiosis (Moldon *et al*. 2008). In fission yeast about 45 % of the genes contain introns. Genes contain from one to more than 10 introns and the introns are small with a mean size of 78 nt (Wood *et al*. 2002). Thus, we reason that in general removal of introns in fission yeast is constitutive by default and, if splicing is coupled with transcription the spliceosomes need to be activated at transcribed loci after the splicing apparatus has recognized the introns to be spliced out.

We suggested that phosphorylation of Prp1 by Prp4 kinase plays a role within the described scenario: either the phosphorylation by Prp4 kinase is part of a mechanism that signals that an intron is occupied by a splicing competent spliceosome in a sense of quality control and/or the phosphorylation is directly involved in inducing the rearrangements for catalysis (Lützelberger *et al*. 2010). Prp1 might act as a kind of proof reading molecule. The phosphorylation status of the N-terminus might be an indicator whether an intron is properly occupied by a competent spliceosome ready to mediate a splicing event.

Intriguingly, in an mammalian *in vitro* splicing system phosphorylation of Prp1 (Prp6/U5-102K) by PRP4 kinase is discussed to stabilize the pre-catalytic B complex consisting of the five snRNPs (Schneider *et al*. 2010).

Based on the results presented in this work and together with our and other research groups findings, we hypothesize that in *S. pombe* the intron definition model is in operation. That is, the information to be recognized as an intron lies predominantly in the intron sequences and the splicing machinery is placed across the introns (Romfo *et al*. 2000). We propose that either the U1 particle first recognizes the 5' splice site of a nascent pre-mRNA tagging an intron. Then, once an intron is tagged by the U1 particle, a tetra-snRNP consisting of U2, U5 and U4/U6 is recruited to the nascent pre-mRNA and a splicing-competent spliceosome (pre-catalytic spliceosome) is formed. Or, based on our recent observations, it is also conceivable that a penta-snRNP recognizes the nascent introns (Figure 4.2).

It has been suggested that *S. pombe* represents the archetype of the pre-mRNA splicing machinery in eukaryotes (Käufer and Potashkin, 2000). The present study supports this prediction. In any case, our hypothesis can be experimentally approached and further analysis will help to provide insight into the complex control mechanisms exerted to express intron-containing genes.

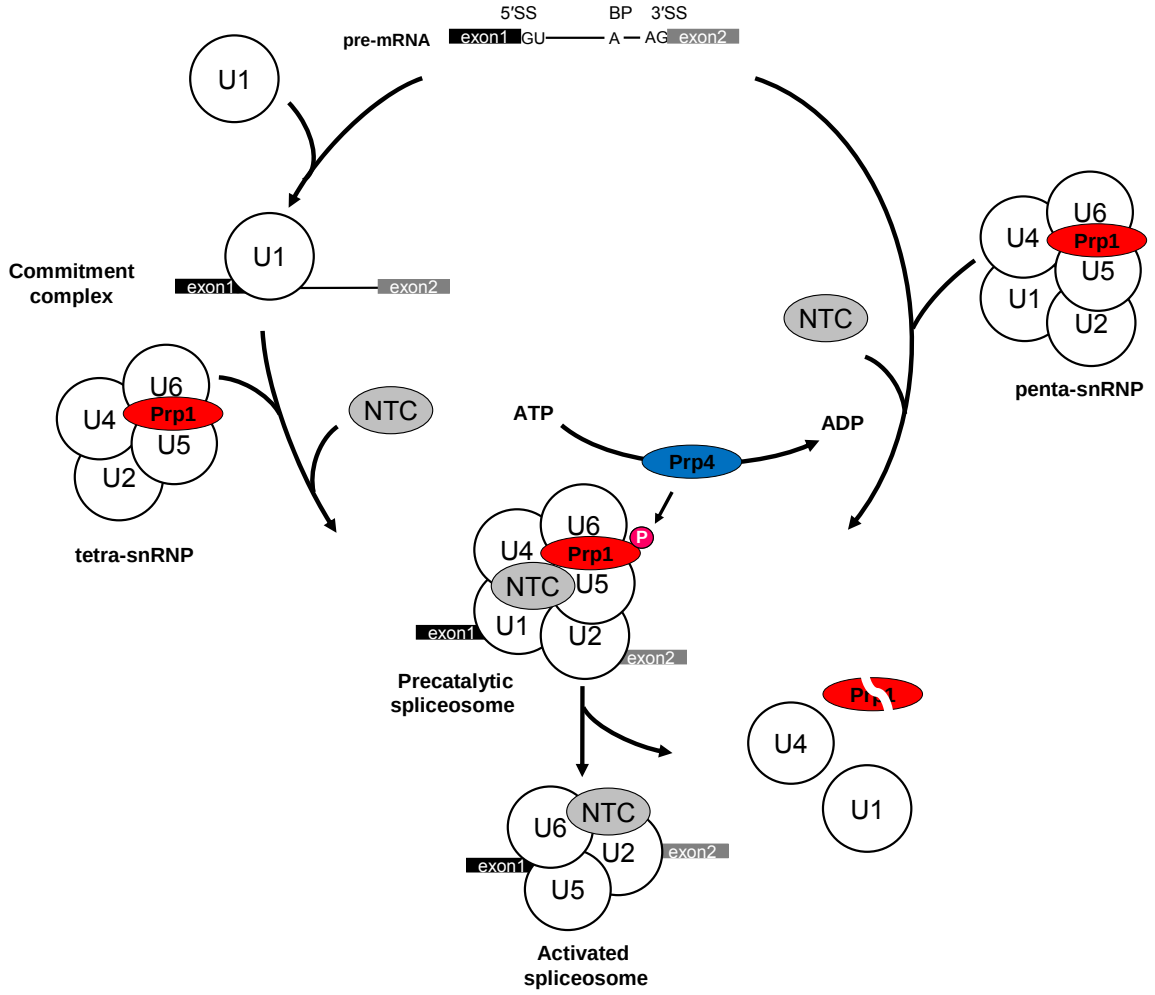

Figure 4.2 Model for the formation of splicing competent spliceosomes. A pre-assembled tetra-snPNP (the left part of the figure) or a penta-snRNP (the right part of the figure) spliceosome is recruited to the pre-mRNA and placed across the intron. 5′SS: 5′ splice site; 3′SS: 3′ splice site; BP: branch point; NTC: nineteencomplex. Explanations see in the text.

5 Materials and methods

5.1 Media

For cultivation of *S.pombe*, media as listed in Table 5.1 were used.

Table 5.1 Media used for cultivation of *S.pombe*

Medium	Composition
Edinburgh minimal medium (EMM)	3 g/L potassium hydrogen phthalate, 2.2 g/L Na_2HPO_2, 5 g/L NH_4Cl, 20 g/L glucose, 20 ml/L 50×solt solution (52,5 g/L $MgCl_2$, 0.735 g/L $CaCl_2$, 2 g/L Na_2SO_4, 50 g/L KCl), 1ml/L solution A1 (500 mg/L H_3BO_3, 40 mg/L $CuSO_4$, 100 mg/L KI, 200 mg/L $FeCl_3$, 530 mg/L $MnSO_4$, 117 mg/L MoO_3, 400 mg/L $ZnSO_4$; as solid or liquid medium. For solid medium 20 g/L agar was used. For cultivation of auxotrophic *S.pombe* strains, minimal medium supplemented with 75 mg/L adenine, 100 mg/L L-histidine, 150 mg/L L-leucine, 50 mg/L L-lysine or 100 mg/L uracil was used.
Yeast extract (YE)	5 g/L yeast extract, 30 g/L glucose, 75 mg/L adenine, 100 mg/L uracil, 150 mg/L L-leucine, 100 mg/L L-histidine, 50 mg/L L-lysine as solid or liquid medium. For solid medium (YEA) 20 g/L agar was used.
Malt extract agar (MEA)	30 g/L malt extract, 75 mg/L adenine, 100 mg/L uracil, 150 mg/L L-leucine, 100 mg/L L-histidine, 50 mg/L L-lysine and 20 g/L agar
Sporulation agar (SPA)	10 g/L glucose, 1 g/L KH_2PO_4, 75 mg/L adenine, 100 mg/L uracil, 150 mg/L L-leucine, 100 mg/L L-histidine, 50 mg/L L-lysine and 30 g/L agar

All media were sterilized by autoclaving for 20 minutes at 121 ºC. After autoclaving, all media except MEA were supplemented with 1ml/L solution A3a (1g/L calcium D (+) pantothenate, 10 g/L nicotinic acid, 10 g/L meso-inositol) and 1 ml/L solution A3b (10 mg/L biotin, 50 % (v/v) ethanol). For selection of nourseothricin resistance strains, minimal or complete medium supplemented with 100 µg/ml nourseothricin was used. For repression of the thiamine repressible *nmt1-8* promoter 0.2 mM thiamine was used.

For cultivation of *E.coli* solid or liquid LB (Luria-Bertani) medium (10 g/L peptone 140, 10 g/L NaCl, 5 g/L yeast extract and 15 g/L agar for solid medium) supplemented with 40 mg/L ampicillin, if required, was used.

5.2 Strains

S.pombe strains used in this work

S.pombe strains used in this work are listed in Table 5.2. The strains indicated were derived from wild-type strains L972 and L975 originally described by Leupold (Leupold U, 1950, 1958). Mating type (h^{-S}, h^{+N}) and relevant alleles are indicated. Gene disruption is designated by a *::* symbol and integration of a vector into *S.pombe* gene is designated by a *int::* symbol.

Table 5.2 *S.pombe* strains used in this work

Strain	Genotype
AG1	h^{+N} *ura4-294 leu1-32 ade6-M216*
AG2	h^{-S} *ura4-294 leu1-32 ade6-M210*
C1	h^{+N} *ura4-294 leu1-32 prp1-127^{ts}*
HE624	h^{-S} *ura4-294 leu1-32 prp1-127^{ts}*
K104	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp101-3*
K116	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp107-1*
K29	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp102-1*
K45	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp106-1*
K54	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp101-2*
K65	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp104-1*
K7	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp101-1*
K84	h^{-S} *ura4-294 leu1-32 prp1-127^{ts} spp105-1*
L972	h^{-S}
L975	h^{+N}
SL150	h^{-S} *ura4-294 leu1-32 ade6-M210 prp1-127^{ts}*
SL151	h^{+N} *ura4-294 leu1-32 ade6-M210 prp1-127^{ts}*
SL156	h^{+N} *ura4-294 leu1-32 ade6-M216 prp1-127^{ts}*
SZE123	h^{-S} *ura4-294 leu1-32 ade6-M216 prp1-127^{ts} spp101-1*
SZE129	h^{-S} *ura4-294 leu1-32 ade6-M216 prp1-127^{ts} spp102-1*
SZE136	h^{+N} *ura4-294 leu1-32 ade6-M210 prp1-127^{ts} spp104-1*
SZE138	h^{+N} *ura4-294 leu1-32 ade6-M216 prp1-127^{ts} spp105-1*
SZE139	h^{+N} *ura4-294 leu1-32 ade6-M210 prp1-127^{ts} spp106-1*
SZE140	h^{-S} *ura4-294 leu1-32 ade6-M216 prp1-127^{ts} spp107-1*

Table 5.2 *S.pombe* strains used in this work (continued)

Strain	Genotype
SL168	*h^{+N} ura4-D18 prp31-E1^{ts}*
UR100	*h^{-S} leu1-32 prp1-4^{ts}*
497	*h^{-S} leu1-32 int::pJK148nmt1-8 prp1 ura4-294 int::pUR19-MycPrp1Δ227-249 prp1::his7 prp31 int::HA-prp31 his7-366*
702	*h^{-S} leu1-32 prp31 int::HA-prp31 prp1 int::Myc-prp1*
714	*h^{-S} leu1-32 int::pJK148nmt1-8 prp1 ura4-294 int::pUR19-MycPrp1Δ227-249 prp1::his7 prp31 int::prp31-CTAP-KanMx6 his7-366*
720	*h^{-S} leu1-32 int::pJK148nmt1-8 prp1 ura4-294 int::pUR19-MycPrp1Δ227-249 prp1::his7 prp31 int::prp31-CTAP-KanMx6 his7-366 prp4 int::HA-prp4*
725	*h^{-S} leu1-32 int::pMLnmt1-8V5lin1 lin1::nat prp31 int::HA-prp31 prp1 int::Myc-prp1*
748	*h^{-S} ura4-294 leu1-32 ade6-M210 prp31 int::HA-prp31 prp1 int::Myc- prp1-127^{ts}*
762	*h^{-S} prp1 int::Myc- prp1-127^{ts} lin1int::lin1-1*

***E.coli* strains used in this work.**

For transformation and amplification of plasmid DNA, *E.coli* strains listed in Table 5.3 were used.

Table 5.3 *E.coli* strains used in this work

Strain	Genotype
DH5α^{TM}	F^{-} φ*80dlacZ*Δ*M15*Δ(lacZYA-argF) Δlac*U196 deoR recA1 endA1 hsdR17* (rK^{-}, mK^{+}) *supE44* λ^{-} *thi-1 gyrA96 relA1*
XL1-Blue	*hsdR17* (rK^{-},mK^{+}) *endA1 supE44 thi-1 recA1 gyrA96 relA1*λ^{-} *lac* F′[proAB^{+} lacI^{q}lacZΔM15 Tn10(tet^{r})]

5.3 Vectors and constructs

pML81-V5

Plasmid pML81-V5 contains the *leu1* gene of *S.pombe,* which can be used for integration into the *leu1-32* locus via homologous recombination. The vector contains the thiamine repressible promoter *nmt1-8* (Maundrell, 1993) followed by a V5 epitope tag, a multiple cloning site and the *nmt1* terminator (Figure 5.1). It can be used for N-terminal tagging of proteins. For selection in *E. coli,* the vector encodes for resistance to ampicillin (*ampR)* (Lützelberger M., AG Käufer, Institut für Genetik, TU Braunschweig).

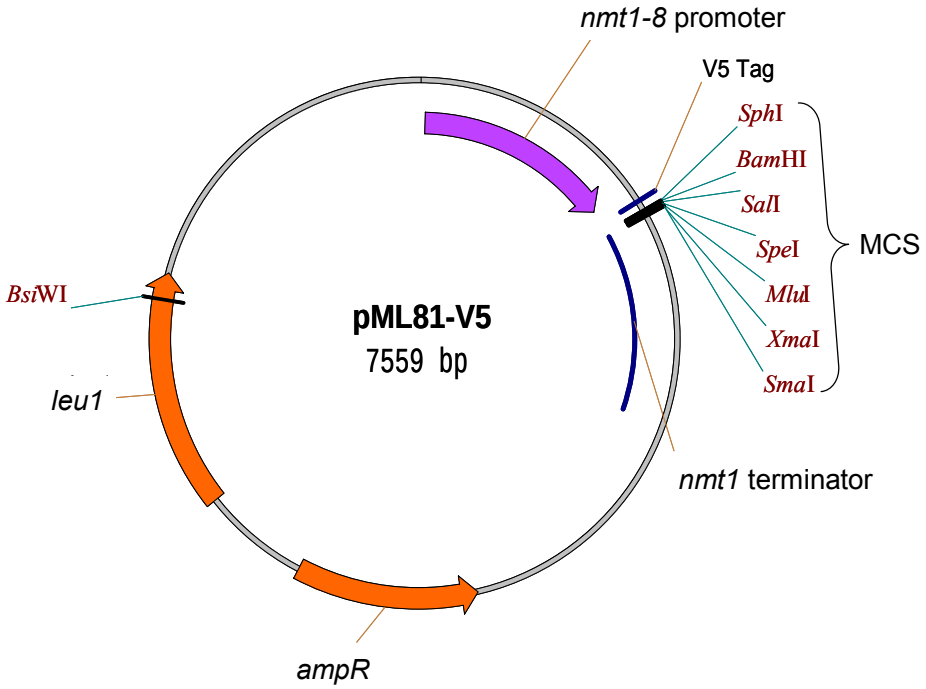

Figure 5.1 Map of the pML81-V5 vector. The vector contains the *leu1* gene, *nmt1-8* promoter and *nmt1* terminator of *S.pombe*. For N-terminal tagging of proteins, pML81-V5 has a V5 epitope tag which is found in the P and V proteins of the paramyxovirus, SV5 (Southern *et al*. 1991). This epitope is followed by a multiple cloning site (MCS) which contains unique recognition sites for restriction endonucleases as indicated. For selection in *E. coli* the vector contains the *ampR* gene conferring resistance to ampicillin. Size of the vector is 7559 bp as shown.

Construction of pML-nmt1-8V5-lin1

In order to place the *lin1* gene under the control of the thiamine-repressible *nmt1-8* promotor, the *lin1* coding sequence (1273 bp) was PCR amplified from the wild-type strain L972 (Table 5.2) using the primer pair lin1-F-BamHI and lin1-R-MluI containing recognition sites for the restriction endonucleases *Bam*HI and *Mlu*I, respectively (Table 5.4). The PCR product was digested with *Bam*HI and *Mlu*I and ligated into pML81-V5 downstream of the V5 tag (Figure 5.2). The resulting plasmid was linearized in the *leu1* gene using *Tth*111I and integrated into the *leu1-32* locus of *S.pombe* (strain 725 [Table 5.2]).

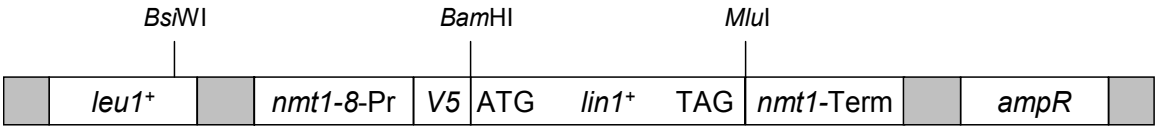

Figure 5.2 Schematic representation of the pML-nmt1-8V5-lin1 construct. The *lin1* coding sequence was PCR amplified and ligated into pML81-V5 downstream of the V5 tag as shown.

Table 5.4 PCR primers used for amplification of the *lin1* coding sequence

Primer	Sequence
lin1-F-BamHI	5′-GAA CAG GAT CCA TGA AGC GTA CGC TCA GAA ACC CT-3′ *Bam* HI
lin1-R-MluI	5′-AAA CTA CGC GTC TAT TTC TTT AAA GAA ACT ACA TCG -3′ *Mlu* I

pFA6a-natMX6

Plasmid pFA6a-natMX6 contains a nourseothricin resistance cassette *natMX6* (Figure 5.3) for gene disruption in *S.pombe*. For selection in *E.coli*, the plasmid contains an ampicillin resistance gene (*ampR*) (Hentges *et al*. 2005).

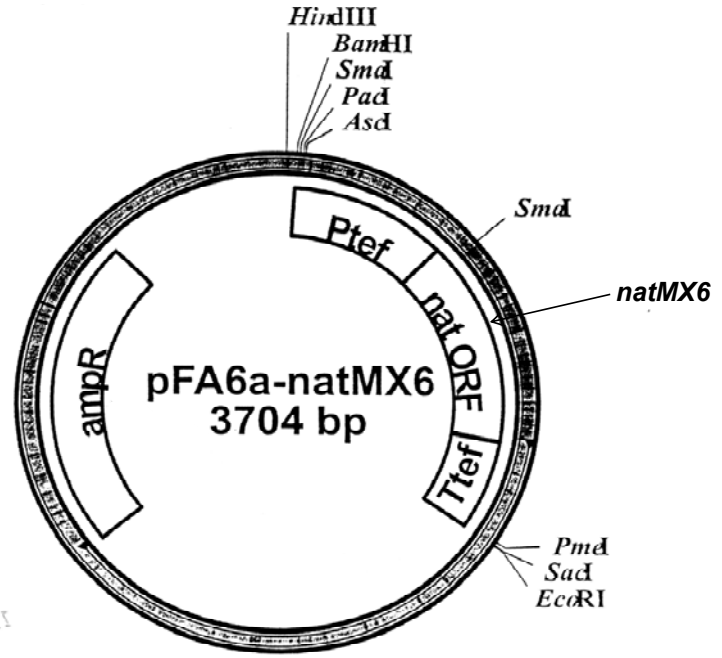

Figure 5.3 Map of the pFA6a-natMX6 plasmid. The plasmid contains a *natMX6* cassette which consist of promoter (P_{TEF}) and terminator (T_{TEF}) sequences of the *Ashbya gossypii* translation elongation factor 1α gene together with the *nat* open reading frame (ORF) of *Streptomyces noursei*. For selection in *E.coli* the vector contains ampicillin resistance gene (*ampR*). Size of the vector is 3704bp as indicated.

Construction of a nourseothricin resistance cassette for *lin1* gene disruption (*5′-lin1-natMX6-lin-3′*)

In order to construct a nourseothricin resistance cassette for *lin1* gene disruption, 5′- and 3′-flanking sequences of the *lin1* gene (about 500 bp and 750 bp, respectively), were amplified by PCR using genomic DNA from wild-type strain L972 as a template. The 5′-flanking sequence (*5′-lin1*) was PCR amplified using the primer pair (5′-homo-lin-like and 5′-homo-lin-like+BamHI, see Table 5.5 and Figure 5.4). The PCR product was digested with *Hind*III and *Bam*HI (the PCR fragment contains a natural *Hind*III site about 70 bp downstream of the 5′-terminus) and ligated into the pFA6a-natMX6 plasmid upstream of the *natMX6* cassette (Figure 5.4). The 3′- flanking sequence (*lin-3′*) was PCR amplified using the primer pair (3′-homo-lin-like+SacI and 3′-homolog-lin-like+EcoRI; see Table 5.5 and Figure 5.4), digested with *Eco*RI and *Sac*I and ligated downstream of the *natMX6* cassette into the pFA6a-natMX6 plasmid harboring *5′-lin1*. The resulting plasmid was cut with *Hind*III and *Sac*I in order to excise a DNA fragment comprising the 5′-flanking sequence of *lin1* followed by *natMX6* and the 3′-flanking sequence of *lin1* (*5′-lin1-natMX6-lin1-3′* cassette). The restriction fragments were separated on an 1 % agarose gel,

the *5′-lin1-natMX6-lin1-3′* cassette was gel eluted and further used for the *lin1* gene disruption in *S.pombe* (strains 725 [Table 5.2]).

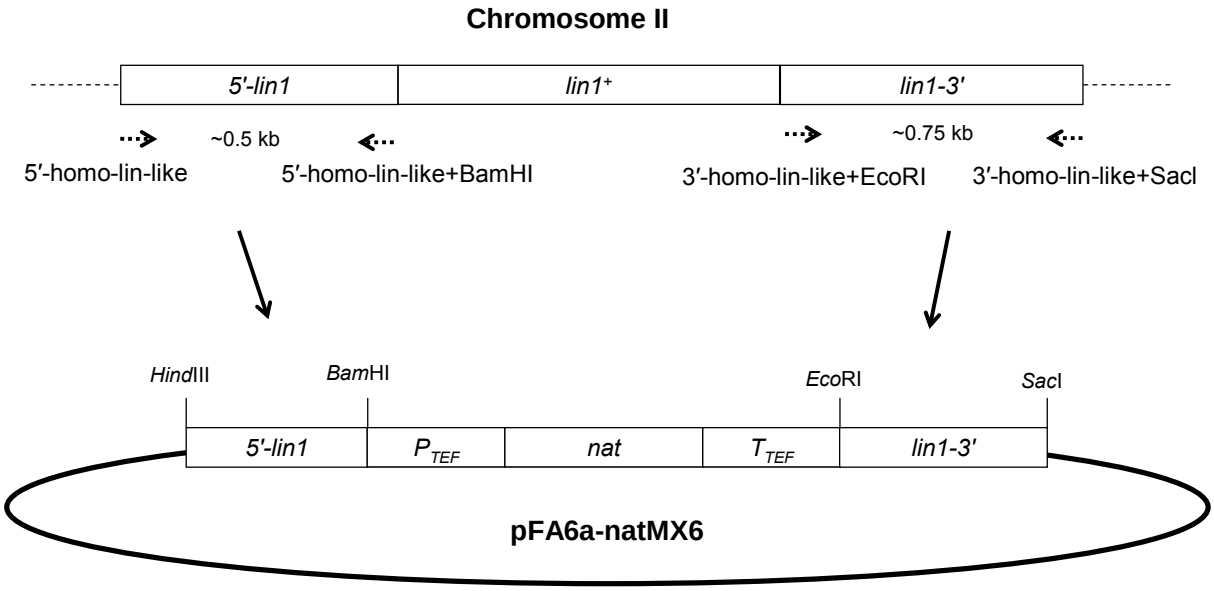

Figure 5.4 5′- and 3′-flanking sequences of the *lin1* gene (*5′-lin1* and *lin1-3′*, respectively) were amplified by PCR using primers pairs (dashed arrows) as indicated. The PCR products were ligated into the pFA6a-natMX6 plasmid upstream and downstream of the *natMX6* cassette as shown. Numbers indicate the size of the PCR products. The *lin1* gene is located on chromosome II in *S.pombe*.

Table 5.5 PCR primers used for construction of a nourseothricin resistance cassette for *lin1* gene disruption

Primer	**Sequence**
5′-homo-lin-like	5′- ATG ACC TTA CTT CGT GCT CCA ACA G-3′
5′-homo-lin-like+BamHI	5′-TCG GAT CCA CAC AAA ATG TAG TTG CAA TTG CGA-3′ *Bam*HI
3′-homo-lin-like+SacI	5′-AAG AGC TCG TGG ATA ATA AAC TAT TTT ATT TCA-3′ *Sac*I
3′-homolog-lin-like+EcoRI	5′-ATG AAT TCT TTT CGT TGA TGA AGT TCA TAT G-3′ *Eco* RI

pAL19

The vector pAL19 is a shuttle vector which can replicate as a high copy number plasmid in *S.pombe* as well as in *E.coli*. This plasmid has a pUC19 (Yanisch-Perron *et al*. 1985) backbone and also contains *LEU2* gene of *S.cerevisiae* and autonomously replicating sequence (*ars1*) for replication in *S.pombe*. It has a multiple cloning site (MCS) located within the *lacZ* gene encoding the N-terminal part of β-galactosidase and therefore can be used for blue-white selection in *E.coli* host strains expressing a defective form of β-galactosidase [mutation Δ*(lacZ)M15*] (Barbet *et al*. 1992).

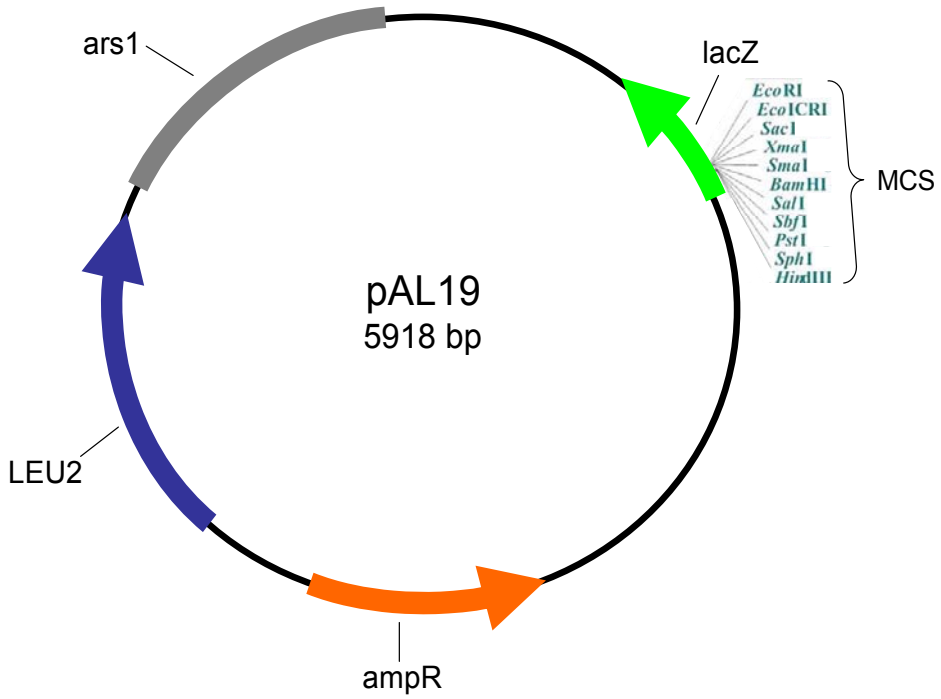

Figure 5.5 Map of the pAL19 vector. The plasmid contains *LEU2* gene of *S.cerevisiae* and *ars1* element of *S.pombe* for replication in fission yeast. The vector also contains truncated *lacZ* gene of *E.coli* which encodes N-terminal part of β-galactosidase (alpha-peptide). A multiple cloning site (MCS) containing unique sites for restriction endonucleases is located within the *lacZ* gene, as indicated. For selection in *E.coli*, the vector contains ampicillin resistance gene (*ampR*). The size of the plasmid is 5918 bp, as indicated.

Construction of pAL19-wt-ptc1 and pAL19-K7-ptc1 plasmids

To construct the plasmid pAL19-wt-ptc1, a DNA fragment containing the coding sequence of the *ptc1* gene (1044 bp) and flanking sequences of about 500 bp both upstream and downstream of *ptc1* was PCR amplified from the wild-type strain L972 (Table 5.2), using

primers ptc1-5′F-HindIII and ptc1-3′R-EcoRI, which contain recognition sites for *Hind*III and *Eco*RI, respectively (Table 5.6). After the PCR fragment was restricted with *Hind*III and *Eco*RI, it was ligated into the multiple cloning site of the pAL19 vector. The pAL19-K7-ptc1 plasmid was constructed as described above, except that the *ptc1* gene was PCR amplified using genomic DNA prepared from strain K7 (Table 5.2). Both plasmids were used for transformation into strain HE624 (Table 5.2).

Table 5.6 PCR primers used for amplification of the *ptc1* gene

Primer	**Sequence**
ptc1-5′F-HindIII	5′-TTC ATA AGC TTA CCA TGA GCC GTT ATT ACC TC-3′ *Hind* III
ptc1-3′R-EcoRI	5′-GGA CAG AAT TCA AAT AAA GAC AAC CCG TAA CTT CA -3′ *Eco* RI

5.4 Construction of strain 725

To construct the strain 725 (Table 5.2), plasmid pML-nmt1-8V5-lin1 linearised with *Tth*111I, which cuts within the *leu1* gene, was transformed into strain 702 (Table 5.2). Leucine prototrophic transformants were tested for integration of the pML-nmt1-8V5-lin1 construct into the *leu1-32* locus by PCR using the primer pair (OL82/OL83, see Table 5.7 and Figure 5.6 A). OL82 binds to the genomic region upstream of the *leu1* locus and OL83 binds to the vector sequence (Figure 5.6 A). In case of correct integration, a PCR product of about 2.3 kb is detected. Expression of V5 tagged Lin1 was confirmed by western analysis with anti-V5 antibodies.

Next, in the transformants harboring *nmt1-8* controlled *V5lin1,* the *lin1* locus was disrupted by integration of the *nat* gene using a linearized DNA fragment comprising the 5′-flanking sequence of *lin1* followed by the *natMX6 cassette* and the 3′-flanking sequence of *lin1* (*5′-lin1-natMX6-lin-3′* cassette). Disruption of the *lin1* locus was confirmed by PCR using primer pair (5′-homo-lin-like/ kanP-TEF, see Table 5.7 and Figure 5.6 B). 5′-homo-lin-like binds to the genomic region upstream of the *lin1* locus and kanP-TEF binds to the promoter P_{TEF} sequence within the *5′-lin1-natMX6-lin-3′* cassette (Figure 5.6 B). In case of successful *lin1* disruption, a PCR fragment of about 1.2 kb is obtained.

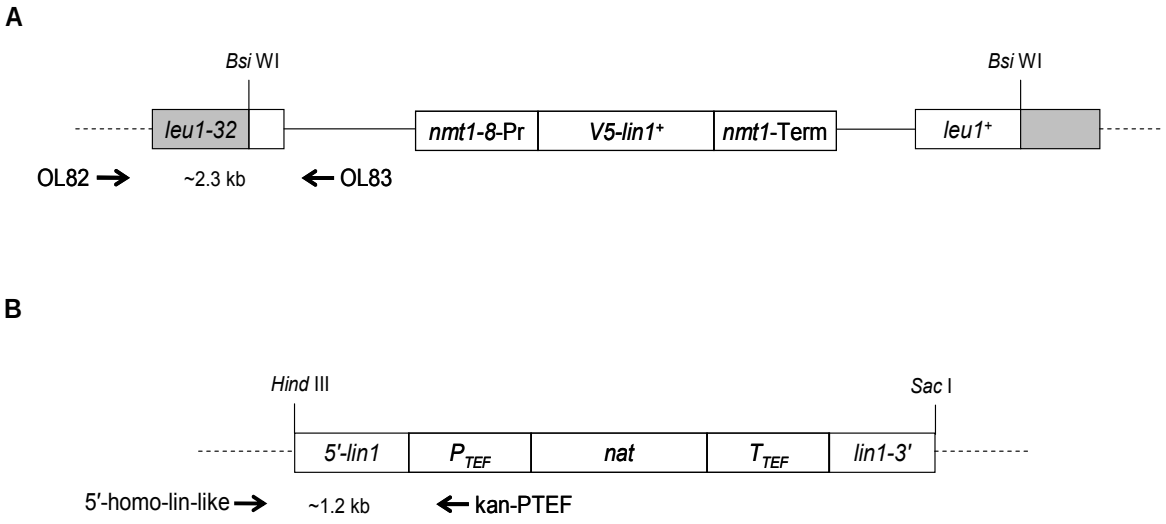

Figure 5.6 Schematic representation of the constructs integrated into chromosome 2 of the strain 725. **A** Integration into the *leu1-32* locus. The pML-nmt1-8V5-lin1 construct was linearized with *Tth*111I and integrated into the *leu1-32* locus (shaded gray). Arrows indicate PCR primers OL82 and OL83. In case of correct integration, a PCR product of about 2.3 kb is detected. **B** *lin1*$^{+}$ gene interrupted with *nat.* The *5′-lin1-natMX6-lin1-3′* cassette was integrated into the *lin1*$^{+}$ locus via homologues recombination. Arrows indicate PCR primers 5′-homo-lin-like and kan-PTEF. In case of correct integration, a PCR product of about 1.2 kb is detected.

Table 5.7 PCR primers used for construction of the strain 725

Primer	**Sequence**
OL82	5′-GCT CCA GGA TAC TTG TAT ATT TCG TTA AA-3′
OL83	5′-CGT TTA CAA TTT CCT GAT GCG GT-3′
5′-homo-lin-like	5′-ATG ACC TTA CTT CGT GCT CCA ACA G-3′
kanP-TEF	5′-ACG AGG CAA GCT AAA CAG-3′

5.5 Classical Genetics with *S.pombe*

Fission yeast is a single celled haploid organism with a short diploid phase in its life cycle. Two haploid cells with opposite mating type can mate with each other by fusing and forming a diploid zygote. The zygote undergoes meiosis and immediately develops into a zygotic ascus containing four haploid spores (Figure 5.7). Upon nitrogen starvation cells of opposite mating type fuse, form diploid nuclei, replicate the DNA and sporulate.

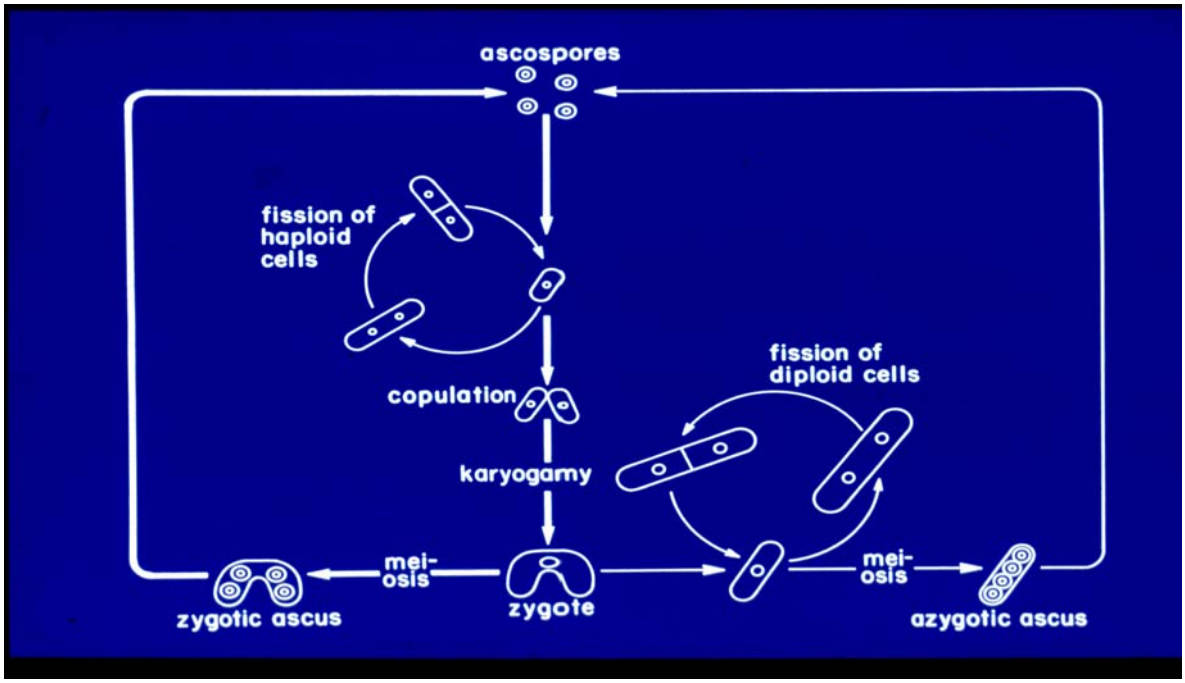

Figure 5.7 Life cycle of *Schizosaccharomyces pombe*. The left part of the figure shows the normal haplontic life cycle. The right part demonstrates the events which take place when zygotes develop to diploid cells. Vegetative cells are haploid. Copulation is triggered by starvation, the zygotes sporulate immediately. However, it is possible to select diploid strain with the help of complementing nutritional markers (the right part) (from King R, "Hand book of genetics", 1974, chapter 25, page 396).

Crosses

S.pombe strains of opposite mating type were streaked on complete agar (YEA) plates and incubated for 2-3 days at 25 ºC. A loopful of freshly growing h^{+N} cells were thoroughly mixed with a loopful of freshly growing h^{-S} cells in an Eppendorf tube containing 100 µl of 0.85 % NaCl solution. The cross was spotted on a malt extract agar (MEA) or a sporulation agar (SPA) plate, shortly dried and then incubated at 25 ºC. Fully formed four spore asci were observed after 2-3 days.

Tetrad analysis

After two days incubating on sporulation medium the asci were straked on a YEA or a minimal agar (EMM) plate for tetrad analysis. To break down the ascus wall the plate was then left for 1-3 hours at 25 °C or overnight at 4 °C. Using micromanipulator (Singer-MSM) four spores of the same ascus were placed at positions with determined coordinates (from A2-A5/A7-A10 to I2-I5/I7-I10) as indicated in Figure 5.8. With this approach 18 tetrads were plated on a standard 9 cm petri dish and the spores were incubated until colonies form at the indicated temperature (see for example Figure 3.2)

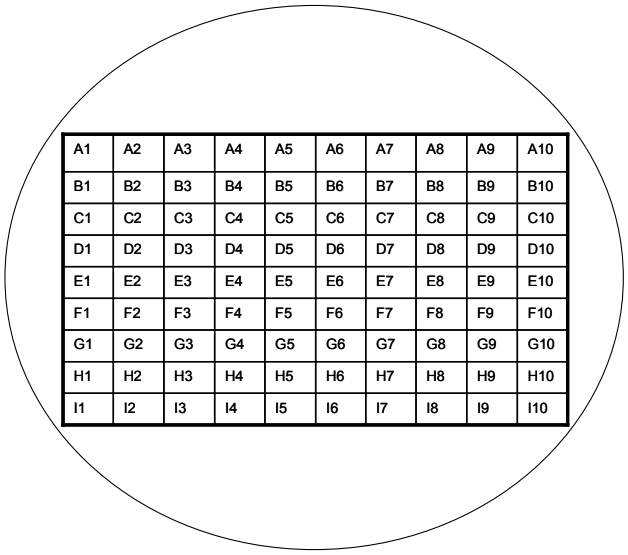

Figure 5.8. Matrix representing a pattern in which spores are placed by the micromanipulator (Singer-MSM).

Isolation of diploid strains

Diploid cells which divide mitotically can be selected based on a mechanism called intragenic complementation. There exist two mutant alleles of *ade6*, called *ade6-M210* and *ade6-M216*. The *ade6* gene encodes a phosophoribosylaminoimidazole carboxylase involved in adenine biosynthesis. Cells carrying *ade6-M210* and *ade6-M216* form red and pink colonies, respectively, on medium with low amounts of adenine supplemented. If both alleles are combined in a diploid cell they can complement each other which lead to the formation of white adenine prototrophic colonies (Gutz *et al*. 1974).

To isolate diploid strains, freshly growing cells of opposite mating type containing *ade6-M210* and *ade6-M216*, respectively, were crossed as described above. Mixed cells were spotted on a SPA plate, shortly dried, incubated for 10 hours at 25 ºC and then examined under a microscope for presence of zygotic asci. After 10, 12, 14 and 16 hours of incubation, cell material of the cross was streaked on EMM agar plates supplemented with

uracil and leucine but not with adenine and incubated for 5 days at 25 ºC. After 5 days of incubation large white colonies and smaller red and pink colonies were formed. Diploid cells are very unstable on EMM and will sporulate if they enter stationary phase. Therefore they were then propagated on YEA medium not supplemented with adenine (YEA-ade) to select for adenine prototrophic diploid cells (white colonies).

Test for dominant and recessive suppressor alleles

Diploid strains homozygous for *prp1-127^{ts}* allele and heterozygous for the *spp101-1*, *spp102-1*, *spp104-1*, *spp105-1*, *spp106-1* and *spp107-1* suppressor alleles were constructed as described above. After streaking the cells on YEA-ade medium, plates were incubated for 5 days at 25 °C and 35 °C, respectively. Growth at 35 ºC indicates the presence of a dominant suppressor mutation, whereas lack of growth suggests that the suppressor mutation is recessive.

Test for growth

30 ml of EMM medium was inoculated with cells from an exponentially growing pre-culture to an OD_{600} of 0.1 and incubated at 25 ºC until OD_{600} of 0.5 had been reached. A serial dilution (1:10, 1:100, 1:1000, and 1:10000) of the cells in 0.85 % NaCl was prepared and 10 µl of each dilution was spotted on EMM agar plates and dried. Plates were then incubated for 7 days at 25 ºC and 35 ºC, respectively.

Genetic mapping of *spp101*

Linkage between *spp101* and the mating type locus (*mat1*) was determined as described in the following.

Strains K7 (*prp1-127^{ts} spp101-1*), K54 (*prp1-127^{ts} spp101-2*) and K104 (*prp1-127^{ts} spp101-3*) were crossed with strain C1 (*prp1-127^{ts}*) (Table 5.2) as discussed above. Tetrads were dissected, spores were grown on YEA medium for 5 days at 25 ºC (permissive temperature) until colonies had been formed, then replicated on YEA medium and incubated at 35 ºC (restrictive temperature) for 5 days. Cells containing the suppressor *spp101* allele in *prp1-127^{ts}* background grow up to colonies, whereas cells containing the temperature sensitive *prp1-127^{ts}* allele in a wild-type background stop dividing at 35 ºC.

To determine a mating type of the growing colonies, the cross was replicated on MEA medium and incubated for 5 days at 25 ºC until colonies had been formed. Then, plates were sprayed with a suspension of a wild-type stain L972 (h^{-S}), incubated over-night at 25

ºC and stained with iodine. Cells of the strain L972 (h^{-S}) mate with h^{+N} cells following by the formation of zygotic asci containing spores. The presence of spores in colonies formed by h^{+N} cells can easily be made visible by treating the plates briefly with iodine vapors (Leupold, 1955). After this treatment, sporulating colonies turn black because the ascospores contain an amyloselike substance which is absent in vegetative cells.

In total 29 asci were dissected and three classed of tetrads were determined: 13 tetrads of parental ditype 'PD' (genotypes of four growing colonies: 2× h^{-S} *spp101*, 2× h^{+N} *spp101*$^{+}$), 3 tetrads of nonparental ditype 'NPD' (2× h^{+N} *spp101,* 2× h^{-S} *spp101*$^{+}$) and 13 tetrads of tetratype 'T' (h^{-S} *spp101,* h^{+N} *spp101,* h^{-S} *spp101*$^{+}$, h^{+N} *spp101*$^{+}$). Then, the recombination frequencies (cM) as suggested by Perkins ($cM = \frac{100}{2}\left[\frac{T + 6NPD}{PD + NPD + T}\right]$) were calculated (Perkins, 1949). This calculation revealed that the distance between *spp101* and the mating type locus (*mat1*) on chromosome 2 is about of 51.6 cM. Since the calculation was done with low numbers of tetrads, it was only used for orientation on the chromosome.

5.6 DNA methods

Transformation of *E.coli*

Preparation and transformation of competent *E.coli* cells

Competent *E.coli* cells were prepared according to a protocol after Mülhardt (2003). 500 ml of LB medium were inoculated with 5 ml of an over-night *E.coli* culture. Cells were incubated for 2-4 hours at 37 ºC until an OD_{600} of 0.4-0.7 had been reached. Then, the culture was chilled on ice and centrifuged for 10 min at 3500 rpm and 4 ºC. After the supernatant was discarded, the pellet was resuspended in 150 ml of ice-cold TFBI buffer (10 mM $CaCl_2$, 100 mM RbCl, 30 mM KOAc [pH 5.8], 15 % glycerin (v/v), 50 mM $MgCl_2$) and incubated on ice for 15 min. The cells were centrifuged for 10 min at 3500 rpm and 4 ºC and then carefully resuspended in 20 ml of TFBII buffer (10 mM MOPS [pH 6.8], 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerin (v/v). Next, the cells were aliquoted (450 µl) in Eppendorf tubes, frozen in liquid nitrogen and stored at -80 ºC until use.

For transformation, an aliquot of competent *E.coli* cells was thawed on ice. After addition of the DNA to transform, the sample was incubated on ice for 40 min, transferred to 42 ºC water bath for 2 min and shifted back to ice for 3 min. After adding 1 ml of LB medium, the cells were incubated for 45 min on a shaker at 37 ºC and centrifuged for 3 min at 5000 rpm. The pellet was resuspended in 100 ml of LB medium and plated on LB agar containing an appropriate antibiotic. Plates were incubated over-night at 37 ºC.

Electrotransformation of *E.coli*

To prepare *E. coli* cells for electrotransformation 1 L of LB medium was inoculated with 10 ml of an overnight culture. Cells were grown to an OD_{600} of 0.5. After the Erlenmeyer flask was chilled on wet ice for 30 minutes, cells were harvested by centrifugation at 4 ºC in a pre-chilled rotor at 4000 rpm for 15 minutes. The pellet was then washed twice with one, and half a culture volume of ice-cold sterile ddH_2O and once with $1/50^{th}$ culture volume of ice-cold 10 % glycerol. The cells were resuspended in $1/500^{th}$ culture volume of 10 % glycerol and aliquoted in 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80 ºC. For electrotransformation 50 µl of competent cells were mixed with 1-4 µl of DNA in low ionic strength medium such as ddH_2O or TE buffer. After the mixture was transferred into a pre-chilled electroporation cuvette, an electric pulse of 12.5 kV/cm was delivered with a time constant of approximately 5 msec using Bio-Rad Gene Pulser. On the apparatus the settings were 2.5 kV (for a 0.2-cm cuvette) with a 25 µF capacitor and a parallel resistance of 200 Ω. Immediately after the pulse, cells were resuspended in 1 ml of

LB medium, transferred to a culture tube and incubated for 45 minutes with aeration at 37 ºC. The cells were then plated on LB agar containing an appropriate antibiotic.

Mini- and Midi- preparations of plasmid DNA from *E.coli*

Plasmid DNA from *E.coli* was prepared with NucleoSpin Plasmid Kit or Nucleobond PC 100 Kit (Macherey-Nagel) according to the manufacturer's instructions. Up to 25 µg of plasmid DNA (mini-preparation) can be isolated with the NucleoSpin Plasmid Kit from a 5 ml over night culture of *E.coli* and up to 100 µg (midi-preparation) can be isolated with the Nucleobond PC 100 Kit from a 50 ml over night culture of *E.coli*.

Construction of genomic DNA libraries

Large scale isolation of chromosomal DNA from *S.pombe*

Chromosomal DNA was isolated using the Blood and Cell Culture DNA Maxi Kit (Qiagen) according to the manufacturer's instructions with the following modifications. 1.5 L of YEL medium was inoculated to an OD_{600} of 0.1 with cells from an exponentially growing pre-culture, incubated for 48 hours at 25 ºC. Then, cells were collected by centrifugation at 5000 rpm for 10 minutes. The supernatant was discarded; cells were washed twice with 300 ml of double distilled H_2O and snap frozen in liquid nitrogen. Frozen cells were grinded in a mortar for 25 minutes until a fine powder was obtained and then resuspended in 30 ml of G2 buffer (Blood and Cell Culture DNA Maxi Kit [Qiagen]). After 100 µl of RNase A (10 mg/ml) and 400 µl of Proteinase K (20 mg/ml) were added, the suspension was incubated for 30 minutes at room temperature and then centrifuged at 6000 rpm and 4 ºC for 15 minutes. The supernatant was further used to isolate DNA as described in the manual to the Blood and Cell Culture DNA Maxi Kit (Qiagen).

Isolated DNA was then precipitated with 2.5 volumes of isopropanol, washed with 500 µl of 70 % ethanol, dried and dissolved in 400 µl of 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA concentration was measured with a spectrophotometer (DU 530, Beckman) at 260 nm.

Partial digestion of genomic DNA with *Sau*3AI

Analytical partial digestion of genomic DNA with *Sau*3AI

In order to establish conditions for partial digestion of genomic DNA with the restriction endonuclease *Sau*3AI (Fermentas), a pilot experiment was performed. The aim of this experiment was to maximize the yield of fragments with a size between 3 to10 kb.

In the experiment, 0.5 µg of genomic DNA were digested with *Sau*3AI in 30 µl of 1× *Sau*3AI restriction buffer (Fermentas). The reactions were performed in nine Eppendorf tubes containing decreasing amounts of *Sau*3AI. For this dilution series of *Sau*3AI with dilution coefficient 1/2 (the first tube contains 5 U of the enzyme, the second tube contains 2.5 U and so on) were prepared. The reactions were incubated for 1 hour at 37 ºC and then transferred to 85 ºC for 10 minutes to inactivate the enzyme. The DNA from each tube was separated in a 0.7 % agarose gel, stained with ethidium bromide and visualized with UV light.

Preparative partial digestion of genomic DNA with *Sau*3AI and separation of DNA fragments in a sucrose gradient

After conditions had been established in the pilot experiment, two preparative restriction reactions with *Sau*3AI were performed. 50 µl of 10 × *Sau*3AI restriction enzyme buffer, 4 U or 8 U of *Sau*3AI, ddH_2O to a final volume of 500 µl were added to 60 µg of genomic DNA. The reactions were incubated for 1 hour at 37 ºC and transferred to 85 ºC for 10 minutes to inactivate the enzyme. The DNA from each reaction was precipitated with 2.5 volumes of 96 % ethanol and 1/10 volumes of 3 M sodium acetate (pH 6.8), dissolved in 100 µl of 1 × TE buffer and pooled. The DNA sample was heated to 68 ºC for 3 minutes, cooled and loaded onto an 11 ml 10-40 % continuous sucrose gradient (10 mM NaCl, 1 mM EDTA [pH 7.5], 10 mM Tris [pH 8.0]). After centrifugation at 22000 rpm and 20 ºC for 22 hours, twenty-six 400 µl-fractions were manually collected from top to the bottom. 10 µl aliquots of each fraction were mixed with 2 µl of 6×DNA loading dye (Fermentas), separated in a 0.7 % agarose gel, stained with ethidium bromide and visualized with UV. Fractions containing DNA fragments which migrate in the range of 3 to 10 kb were then pooled; the DNA was precipitated with 2.5 volumes of 96 % ethanol and 1/10 volumes of 3 M sodium acetate (pH 6.3) and dissolved in ddH_2O.

Partial fill-in of 5′- termini of genomic DNA fragments

Partial digestion of genomic DNA with *Sau*3AI generates DNA fragments with self-compatible four nucleotide-long 5′-overhangs. To prevent self-ligation, a partial fill-in of the 5′-termini was performed. 10 µl of 10 × Klenow Fragment reaction buffer (Fermentas), 1 µl of dATP (2.5 mM), 1 µl of dGTP (2.5 mM), 10 U of DNA polymerase I (Klenow Fragment [Fermentas]) and ddH_2O to a final volume of 100 µl were added to the DNA from the previous step. The reaction was incubated for 1 hour at 37 ºC and the DNA was then precipitated with 96 % ethanol and dissolved in ddH_2O.

Digestion of pAL19 with *Sal*I and partial fill in of 5′-termini

The plasmid pAL19 contains a unique *Sal*I restriction site within its polylinker. To digest pAL19 with *Sal*I (Fermentas), 5 µl of 10 × *Sal*I restriction enzyme buffer (Fermentas), 10 U of *Sal*I, and ddH_2O to a final volume of 50 µl were added to 10 µg of plasmid DNA. The reaction was incubated for 3 hours at 37 ºC. Afterwards, the DNA was precipitated with 2.5 volumes of 96 % ethanol and 1/10 volumes of 3 M sodium acetate (pH 6.3) and dissolved in ddH_2O. Linearized pAL19 plasmid was further used for a partial fill in of 5′-termini. 10 µl of 10 × Klenow Fragment reaction buffer, 1 µl of dTTP (2.5 mM), 1 µl of dCTP (2.5 mM), 10 U of DNA polymerase I (Klenow Fragment), and ddH_2O to a final volume of 100 µl were added to the plasmid DNA from the previous step and the reaction was incubated for 1 hour at 37 ºC. The DNA was then precipitated again with 96 % ethanol and dissolved in ddH_2O.

Ligation of genomic DNA fragments into pAL19

The ligation reaction was performed with T4 DNA ligase (Fermentas). 300 ng of partially filled-in genomic DNA fragments, 1 µl of 10 × T4 DNA ligase reaction buffer (Fermentas), 1 U of T4 DNA ligase and ddH_2O to a final volume of 10 µl were added to 100 ng of linearized and partially filled-in pAL19 plasmid. The reaction was incubated over night at 14.8 ºC and then heated to 65 ºC for 10 minutes to inactivate the enzyme.

Amplification of the genomic DNA library

To amplify the genomic DNA library, the ligation reaction from the previous step was transformed into the *E.coli* strain XL1-Blue by electroporation. Cells were plated on LB medium containing 40 µg/ml ampicillin, 0.1 mM X-Gal, 0.2 mM IPTG and incubated over night at 37 ºC. From ten randomly picked white colonies plasmid DNA was isolated and

restriction analysis with *Hind*III and *Eco*RI was performed. The DNA fragments were separated in a 1 % agarose gel and the average size of the genomic DNA inserts was estimated.

All transformants were then washed from plates with 0.85 % NaCl solution and used for DNA preparation. The plasmid DNA was prepared using the Nucleobond PC 100 Kit (Macherey-Nagel) according to the manufacturer's instructions.

Screening of the genomic DNA library

The genomic library produced in the previous step was transformed into strain HE624 (Table 5.2). After transformation, the cells were plated on EMM agar supplemented with uracil (EMM+ura), incubated over night at 25 ºC, shifted to 35 ºC and incubated for 6-10 days. Growing colonies were streaked on EMM+ura plates and incubated at 35 ºC to confirm their ability to grow at 35 °C. Plasmid DNA was isolated from growing cells and transformed into the *E.coli* strain XL1-Blue by electrotransformation. After plasmids were recovered from *E.coli*, their inserts were analyzed by cleavage with *Hind*III and *Eco*RI. The DNA fragments were separated in a 1 % agarose gel containing ethidium bromide and visualized with UV.

To test which of the isolated plasmids were able to rescue the growth defect the strain HE624, they were retransformed. After transformation, the cells were plated on EMM+ura medium, incubated over night at 25 ºC, shifted to 35 ºC and incubated for 6-10 days.

Transformation of *S.pombe*

Transformation of *S.pombe* was performed by the LiOAc- method as described by Ito *et al*. (1983). 100 ml of EMM medium was inoculated with *S.pombe* cells from an exponentially growing pre-culture to an OD_{600} of 0.1 and incubated at 25 ºC or 30ºC until an OD_{600} of 0.6-0.9 had been reached. Cells were harvested by centrifugation at 5000 rpm for 3 minutes. The supernatant was discarded and cells were washed twice with 40 ml of sterile ddH_2O and once with 10 ml of TE/LiOAc solution (100 mM LiOAc, 1 × TE buffer [10 mM Tris, 1 mM EDTA, pH 8.0]). The cells were then resuspended to 2×10^9 cells/ml in TE/LiOAc solution and incubated for 15 minutes at 25 ºC or 30 ºC with gentle agitation. For each transformation reaction 100 µl of cells were placed into a 2 ml Eppendorf tube. After 10 µl of freshly denatured salmon sperm DNA (10 mg/ml) and the DNA to transform (1.5-2 µg) were added, samples were incubated for 30 minutes at 25 ºC or 30 ºC.

Afterwards, to each transformation 500 µl of TE/LiOAc/PEG (100 mM LiOAc, 1 × TE buffer, 40 % PEG) solution were added, mixed thoroughly and incubated for 30 minutes at 25 ºC or 30 ºC. The transformation reactions were shifted to 42 ºC for 20 minutes (heat shock) and centrifuged for 1 minute at 6000 rpm. After the supernatant was discarded, cells were resuspended in 100 µl of sterile ddH_2O and plated on EMM agar plates. Plates were then incubated for 4-7 days at 25 ºC or 30 ºC.

Small scale isolation of chromosomal DNA from *S.pombe*

Chromosomal DNA from *S.pombe* was isolated according to a protocol of Hoffmann and Winston (1987). 4 ml of YEL or EMM medium was inoculated with freshly growing cells and incubated for 24 hours at 25 ºC or 30 ºC. Cells were harvested by centrifugation at 13000 rpm for 1 minute, washed with 1 ml of sterile ddH_2O, centrifuged as above and the supernatant was discarded. After 200 µl of Buffer 1 (2 % Triton X-100 (v/v), 1 % SDS (w/v), 100 mM NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA), 200 µl of PCIA (phenol/chloroform/isoamyl alcohol 25:24:1) solution and 0.4 g of glass beads were added, the cells were lysed using a bead beater (Fast Prep [FP120]) for 45 seconds at 4500 rpm, 4 ºC. After 200 µl of 1 × TE buffer were added, the samples were centrifuged at 13000 rpm for 5 minutes and the upper aqueous phase of the supernatant was placed in a new Eppendorf tube. The DNA was precipitated with ethanol and centrifuged immediately at 13000 rpm for 10 minutes. The precipitate was then resuspended in 400 µl of 1 × TE buffer containing 75 µg/ml of RNaseA and incubated for 30 minutes at 37 ºC. Afterwards, the DNA was extracted with 400 µl of PCIA solution, precipitated with 40 µl of 3M sodium acetate (pH 6.3) and 1 ml of 96 % ethanol and centrifuged at 13000 rpm for 10 minutes; the pellet was then washed with 500 µl of 70 % ethanol, dried and resuspended in 30 µl of 1 × TE buffer.

Restriction digestion of DNA with restriction endonucleases

Restriction digestion of double-stranded DNA was performed with restriction endonucleases supplied by Fermentas. For digestion of 1 µg DNA, 1 U of an enzyme was used. Reaction conditions were set according to the manufacturer's instructions.

Ligation of DNA

The ligation of DNA fragments into a vector was performed with T4 DNA Ligase (Fermentas). Reactions were performed in a final volume of 20 µl with 100-200 ng of vector DNA and 1 U of ligase. The molar ratio of vector to insert DNA was adjusted to 1: 3. The reactions were incubated over night at 14.8 ºC or for 1 hour at 22 ºC.

Polymerase Chain Reaction (PCR)

In this work several different DNA polymerases were used for PCR. Both, *Pfu* polymerase from *Pyrococcus furiosus* (Fermentas) and *Herculase II Fusion DNA Polymerase* (Stratagene) possess a "proof reading" activity and therefore they are characterized by a relatively low error rate. These polymerases were applied for amplification of DNA fragments which were subsequently used for cloning or sequencing. In other cases *TaKaRa Ex Taq* (Takara Bio Inc) was used. All reactions were mixed according to the manufacturer's instructions. In general, DNA was amplified with 25-35 cycles of 94 ºC for 20 s, 53-61 ºC (depending on annealing temperature of primers used) for 20 s and 72 ºC for 30 s - 6 min (depending on the length of a DNA fragment to amplify) in a thermo cycler (Mastercycler personal, Eppendorf).

Amplification of *spp101-1* suppressor candidates of *prp1-127^{ts}* by PCR

The *spp101-1* suppressor candidates (Table 3.1) of *prp1-127^{ts}* were PCR amplified using genomic DNA isolated from strain K7 (Table 5.2) as a template. DNA polymerase *TaKaRa Ex Taq* (Takara Bio Inc) was used in the reactions. All reactions were mixed according to the manufacture's instructions. DNA was amplified with 35 cycles of 95 ºC for 20 s, 55 ºC for 20 s and 72 ºC for 5 min in a thermo cycler (Mastercycler personal, Eppendorf). For amplification of the candidate genes, primer listed in Table 5.8 were used.

Table 5.8 Primers used for amplification of *spp101-1* suppressor candidates

Primer	**Sequence**
sf3b14-F	5′-CATTGCCAAACAAGAGTATAAGTTA-3′
sf3b14-R	5′-AGCTTTAGTGTGTTCCGTAAT-3′
lsm3-F	5′-CCATGAAATGAAGAACTGCGACA-3′
lsm3-R	5′-GCCTATATATGATGATACGCTTGTT-3′
cwc16-F	5′-TCGCATAACGGTCCTTTCCCTACTC-3′,
cwc16-R	5′-GTTGACGTGATGCCCATTGACTGT-3′
sme1-F	5′-CAATGTAAAGCGCATAGTAGTCTGT-3′
sme1-R	5′-TCGTCGAAAGAACCTGAACAATCC-3′
smd3-F	5′-CCGGTAATTATCATCTCGATATT-3′
smd3-R	5′-TGCTTTCTTAACATGGCTTCGCTAT-3′
prp38-F	5′-GGCAAATCTCGATGAGTAGCTTAAA-3′,
prp38-R	5′-ACACGGAAACTGACGGAATTGAG-3′
cdc28-F	5′-GAAGGAAGTTGGGAAGAGTCGC-3′
cdc28-R	5′-ATATTTAGAAATGCTTGGGAACGAG-3′
pop3-F	5′-TTTCTGTTATACGTCTTTCGGAACC-3′
pop3-R	5′-TCAAATTCGCTTCTTCGCTAACC-3′
cwf7-F	5′-ATTGAGTCTTGGCGAGCACTA-3′
cwf7-R	5′-TTTCGGTGAGGTCACTGAGGGTATG-3′
lin1-F	5′-ATGACCTTACTTCGTGCTCCAACAG-3′
lin1-R	5′-ATGAATTCTTTTCGTTGATGAAGTTCAT ATG-3′
prp5-F	5′-ATGTTTGGCCTGGCTATGTA-3′,
prp5-R	5′-AGGTTGAAATCTACGCTTGTTAGTT-3′
msl1-F	5′-GTGATTTGGACATTTCGGTTCACT-3′
msl1-R	5′-ACAATTCCATCAAATGCGTTAAAGT-3′

Separation of DNA fragments by gelelectrophoresis

DNA fragments were separated in 0.7 %, 1 % or 3 % agarose gel containing 0.2 µg/ml of ethidium bromide and visualized with UV light. For loading, DNA samples were mixed with 1/6 sample volume of 6 × DNA loading dye (Fermentas). As a running buffer 1 × TBE (100 mM Tris, 100 mM H_3BO_3,

2 mM EDTA, pH 7.4) was used. Separation of DNA was performed for 15-45 minutes at 120-200 V. DNA fragments (GeneRuler^{TM} 1 kb DNA ladder [#SM0311], MassRuler^{TM} High Range DNA Ladder [#SM0393], GeneRuler^{TM} 50 bp DNA Ladder [#SM0371]; Fermentas) with known size were used as size markers.

Sequencing of DNA

The chain-termination method developed by Sanger *et al*. (1977) was used for sequencing of DNA. The Cycle-Sequencing Kit (Perking Elmer Applied Biosystem) was utilized for sequencing reaction. For endlabeling of DNA fragments four different fluorescently labeled 2′,3′- dideoxynucleotides were used. This approach permits sequencing in a single reaction. ABI Prism^{TM} Genetic Analyzer was used to perform capillary electrophoresis for size separation, detection, recording of fluorescence and data output as fluorescence peak trace chromotograms.

Sequencing reactions were performed in a final volume of 10 µl. 2 µl of premix (dNTPs, ddNTPs, DNA-polymerase, $MgCl_2$, buffer), 2 µl of 5 × reaction buffer, 5 pmol of a primer, 0.5 µg of plasmid DNA or a PCR product were mixed and the reaction was incubated in a thermo cycler (Mastercycler personal, Eppendorf) for 25 cycles of 96 ºC for 30 seconds, 46 ºC for 15 seconds and 60 ºC for 4 minutes. After 40 µl of ddH_2O were added to the reaction, the DNA was precipitated with 5 µl of 3 M sodium acetate (pH 6.3), 1 µl of dextranblue (20 mg/ml) and 125 µl of 96 % ethanol, washed with 70 % ethanol, dried, dissolved in 12,5 µl of TSR (template suppression reagent)-buffer, and incubated for 2 minutes at 90 ºC. The sample was transferred to a sample tube and stored at 4 ºC before analysis.

5.7 RNA methods

Isolation of RNA

Isolation of RNA from *S.pombe*

RNA was isolated form *S.pombe* according to a protocol published by Nischt *et al*. (1986). Cells were harvested from 10-50 ml of exponentially growing *S.pombe* culture by centrifugation for 5 min at 3000 rpm. The pellet was washed with 20-50 ml of sterile ddH_2O and centrifuged as above. After the supernatant was discarded, the cells were frozen in liquid nitrogen and stored at -80 ºC until use. For isolation of RNA, 400 µl of LETS buffer (100 mM LiCl, 10 mM Tris [pH 7.4], 10 mM EDTA) containing 1 % SDS (w/v) and 0.3 g of glass beads were added to the sample. Next, the cells were lysed in a bead beater (Fast Prep FP120) at 4500 rpm for 30 second and immediately transferred on ice. After adding 400 µl of LETS buffer containing 0.2 % SDS (w/v), the sample was shortly vortexed and centrifuged for 10 min at 13000 rpm and 4 ºC to remove excessive foam. Then, 800 µl of PCIA solution (phenol/chloroform/isoamylalcohol [25:24:1]) solution were added to the sample; it was shortly vortexed and centrifuged for 10 min at 13000 rpm and 4 ºC. The upper aqueous phase was transferred to a new Eppendorf tube and extracted twice with 800 µl of PCIA solution. Afterward, the upper aqueous phase was transferred into a glass tube. To increase the yield, RNA was re-extracted form the lysed cells. 800 µl of LETS buffer containing 0.2 % SDS (w/v) were added to the lysed cells, the sample was shortly vortexed and spun as above. Then, the upper aqueous phase was transferred to a new Eppendorf tube and extracted two more times with 800 µl of PCIA solution. Next, the upper aqueous phase was pooled into a glass tube containing material from the first extraction. To precipitate RNA, 5 ml of 96 % ethanol and 40 µl of 5 M LiOAc were added and the sample was centrifuged for 20 min at 13000 rpm and 4 ºC. The pellet was washed with 5 ml of 70 % ethanol, dried in a vacuum concentrator (Speed Vac Plus SC110A), and dissolved in 400 µl of ddH_2O. Then, RNA was precipitated again by adding 2 ml of 96 % ethanol and 16 µl of 5 M LiOAc. After the sample was centrifuged for 20 min at 13000 rpm and 4 ºC, the pellet was washed with 2 ml of 70 % ethanol, dried and dissolved in 50-100 µl of ddH_2O. The concentration of the purified RNA was measured with a spectrophotometer at 260 nm.

Isolation of RNA from immunoprecipitates or TAP eluates

For isolation of RNA, immunoprecipitates or TAP eluates were mixed with one volume of Phenol/Chloroform/Isoamylalcohol (25:24:1) solution and vortexed. After the samples were centrifuged for 10 minutes at 13000 rpm and 4 ºC, the upper aqueous phase was collected and extracted with one volume of chloroform. The RNA was then precipitated with 2.5 volumes of 96 % ethanol and 1/10 volume of 3 M sodium acetate (pH 6.3). The samples were incubated over night at -20 ºC and centrifuged for 20 minutes at 13000 rpm and 4 ºC. The pellet was washed with 200 µl of ice-cold ethanol, dried briefly in a vacuum concentrator and dissolved in nuclease-free water. To detect U snRNAs the RNA was used for reverse transcriptase (RT) –PCR analysis as described.

Reverse transcriptase –PCR

Prior to Reverse transcriptase (RT)-PCR, the RNA extracted from TAP eluates or from anti-V5 antibody immunoprecipitates was treated with RQ1 RNase-free DNase (Promega) in order to eliminate possible DNA contaminants. Each microgram of RNA was treated with 0.1 U RQ1 DNase in 1×reaction buffer (40 mM Tris–HCl (pH 8.0), 10 mM $MgSO_4$ and 10 mM $CaCl_2$). After incubation for 30 min at 37 ºC, the RQ1 DNase was inactivated by the addition of 2 mM EGTA (pH 8.0) and heating to 65 ºC for 10 min. The RNA was reverse transcribed, and the cDNA amplified with MasterAmpTM Tth polymerase (Epicentre) using the 'single-tube' protocol according to the manufacturer's instructions. In brief, the RNA was first incubated in presence of 1×reaction buffer (20 mM $(NH_4)_2$ SO_4, 50 mM Tris–HCl, pH 9.0), 3 mM $MgCl_2$, 400 mM dNTP (each), 1×MasterAmpTM PCR enhancer, 0.5 mM $MnSO_4$, 0.5 mM of forward and reverse primer and 1.25U MasterAmpTM Tth polymerase for 20 min at 65 ºC. Then, the cDNA was amplified with 18–25 cycles of 94 ºC for 30 s, 54 ºC for 30 s and 72 ºC for 25 s. The PCR products were separated on a 3 % agarose gel using 1×TBE as a running buffer and stained with ethidium bromide. For amplification of the U snRNAs primers listed in Table 5.9 were used. For amplification of transcripts revealing spliced and unspliced versions of the ribosomal protein genes *rpl29* and *rps27* in Table 5.10 were used.

Table 5.9 Primers used for amplification of the snRNAs

Primer	**Sequence**
U1F	5′-CGGGATCCACTTACCTGGCATGAGTTTC-3′
U1R	5′-ACGCGTCGACTGCCCCAAATGAGGGACG-3′
U2F	5′-ATATGGATCCATTCTCTCTTTGCCTTTTG-3′
U2R	5′-ATATGTCGACATTCGGCGTCGCTTGCCA-3′
U4F	5′-ATATGGATCCATCTTTGTGCACGGGTATT-3′
U4R	5′-ATATGTCGACAGTTGGTTTCCAAATATTCC-3′
U5F	5′-ATATGGATCCATAATCCGTCAAAGCACTTT-3′
U5R	5′-ATATGTCGACTTTCAAGAAAAAGATTACAAAAA-3′
U6F	5′-ATATGGATCCGATCTTCGGATCACTTTGG-3′
U6R	5′-ATATGTCGACAAAATGGGTTTCTCTCAAT-3′

Table 5.10 Primers used for amplification of *rpl29* and *rps27*

Primer	**Sequence**
rpl29F	5′-ATGGCCAAGTCGAAGAATCATACTA-3′
rpl29R	5′-TTGGTTGCGGCGGAACTTA-3′
rps27F	5′-TACCCCGAGCAATTCACTAACA-3′
rps27R	5′-ACAACGGTTTGAGCATGAGAGA-3′

5.8 Biochemical methods

Protein extracts

Protein extracts were prepared according to a protocol published by Moreno *et al*. 1991. Cells harvested from 50 ml of an exponentially growing culture were washed with 1 ml of STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EGTA, 1 mM NaN_3, pH 8.0) and centrifuged for 1 minute at 13000 rpm. The supernatant was discarded and the cell pellet was snap frozen in liquid nitrogen and stored at -20 ºC until use. To lyse the cells, 250 µl of HB buffer (25 mM MOPS, 60 mM β-Glycerophosphate, 15 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4, 15 mM $MgCl_2$, 150 mM NaCl, pH 7.2) containing protease inhibitors (1 × Complete Mini EDTA-free, Roche Diagnostics, 4 µg/ml leupeptin, 1.3 mM benzamidine, 1 mM PMSF) and 0.5 g of 0.3 mm glass beads were added to the cell pellet. The cells were immediately lysed in a bead beater (Fast Prep FP120, Savant) with three cycles of 6500 rpm for 30 second. Between the cycles samples were chilled on ice for 5 minutes. To improve the lysis procedures, a bead beater (Precellys 24, Peqlab Biotechnologie GmbH) equipped with a cooling unit was utilized. In the cooling unit, compressed air is chilled with liquid nitrogen and then pumped directly into the bead beater. This allows to maintain constant temperature during the lysis. Using this setup, the cells were lysed (Precellys 24, Peqlab Biotechnologie GmbH) with three cycles of 6500 rpm for 30 second and a break of 30 seconds between the cycles. In the course of the lysis, temperature between -5 and -10 ºC was maintained. After cell disruption, the glass beads were washed once with 250 µl of HB buffer containing protease inhibitors. The lysate was cleared by centrifugation for 15 minutes at 13000 rpm and 4 ºC. The supernatant was either immediately used (for glycerol gradient centrifugation) or frozen in liquid nitrogen and stored at -80 ºC until use.

Determination of protein concentrations (Bradford assay)

The Bradford assay is based on a shift of maximum absorbance spectrum of the dye Coomassie Brilliant Blue G 250 from λ=465 nm to λ=595 nm following binding to proteins (Bradford *et al*. 1976).

A calibration curve was generated using protein samples containing known amounts of bovine serum albumine (2.5-20 mg/ml). 100 µl of the protein standard solution were mixed with 900 µl of Bradford reagent (0.08 mM Coomassie Brilliant Blue G 250, 2.85 % (v/v) EtOH, 8.78 % (v/v) phosphoric acid) and incubated for 5 minutes at room temperature. Absorbance of the samples was measured with a spectrophotometer (Beckman DU 530) at 595 nm. Then, the amount of BSA measured was expressed along the x-axis and

corresponding absorbance was expressed along the y-axis. The calibration curve for Bradford assay remains linear only from about 2.5 µg to 15 µg. If absorbance of unknown protein sample falls outside of this range, the margin of error becomes very high. In order to remain in the linear range, a sample was four times diluted and used to measure a protein concentration. The absorbance was measured at 595 nm and the protein concentration in the sample was determined using the calibration curve.

SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated based on their electrophoretic mobility using SDS- gel electrophoresis (Laemmli *et al*. 1970). To prepare a 50 mm x 130 mm gel, a 4 % stacking gel (4 % acrylamide/bisacrylamide (37.5:1), 31.25 mM Tris, 0.025 % (w/v) SDS, 0.1 % (w/v) APS, 0.1 % (v/v) TEMED, pH 6.8) was cast over a 10 % separating gel (10 % acrylamide/bisacrylamide (37.5:1), 93.75 mM Tris, 0.025 % (w/v) SDS, 0.1 % (w/v) APS, 0.1 % (v/v) TEMED, pH 8.8) Before loading, the protein extract was mixed with 2 × SDS sample buffer (4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20 % (v/v) glycerin, 200 mM β-mercaptoethanol, 100 mM Tris [pH 6.8]), incubated for 10 minutes in a boiling water bath and shortly spun. Proteins were concentrated on a stacking gel at 100V and then separated on a separating gel at 250 V. 1 × glycine buffer (192 mM glycine, 0.1 % (w/v) SDS, 25 mM Tris, pH 8.3) was used as a running buffer. Proteins with known molecular weight (Prestained Protein Molecular Weight Marker [#SM0441]; Fermentas) were used as a marker.

Western Blotting

For western blotting, proteins were separated by SDS-PAGE and transferred onto Protran nitrocellulose membrane (Whatman) by semi-dry blotting which was performed in a blotting apparatus supplied by Phase GmbH (Pegasus, model S). The transfer was performed at 2.3 mA/cm^2 for 60 minutes (1.5 mm gel) in a transfer buffer (6 mM Tris, 48 mM glycine, 19.6 % (v/v) methanol). After blocking over night with 10 % non-fat milk powder in 1×TBS (150 mM NaCl, 100 mM Tris HCl, pH 7.5) at 4 ºC, the membrane was incubated with appropriate primary antibodies for 1 hour or over night at 4 ºC. After washing four times with 1×TBS, the membrane was incubated with secondary HRP-conjugated anti-mouse antibodies or with HRP-conjugated anti-rabbit antibodies for 1 hour at 4 ºC and washed four times with 1×TBS. Protein bands were visualized by

chemiluminescence (SuperSignal West Pico or SuperSignal West Femto substrate, Thermo Scientific) according to the manufacturer's instructions. Antibodies listed in Table 5.11 were used in the analysis.

Table 5.11 Antibodies used in this work

Antibody	**Host**	**Supplier**	**Clone**	**Catalog number**	**Dilution**
anti-HA monoclonal IgG antibodies	mouse	Hiss Diagnostics	16B12	MMS-101R	1:1000 1:2000
anti-Myc monoclonal IgG antibodies	mouse	Hiss Diagnostics	9E10	MMS-150R	1:1000
anti-V5 monoclonal IgG antibodies	mouse	Invitrogen		R960-25	1:1000
anti-Prp31 polyclonal antibodies	rabbit	Eurogene		custom made	1:1000
anti-Prp1 polyclonal antibodies	rabbit	Eurogene		custom made	1:1000
anti-TAP monoclonal IgG antibodies	mouse	Sigma		I5381-1MG	1:1000
HRP-conjugated anti-mouse IgG antibodies	goat	Jackson ImmunoResearch Laboratories, Inc		115-035-003	1:1000 1:20000 1:40000 1:60000 1:120000
HRP-conjugated anti-rabbit IgG antibodies	goat	Jackson ImmunoResearch Laboratories, Inc		111-035-003	1:1000 1:60000 1:120000

Glycerol gradient centrifugation

To prepare a 10-30 % continuous glycerol gradient (25 mM MOPS [pH 7.2], 15 mM EGTA, 15 mM $MgCl_2$, 50 mM NaCl, 60 mM β-glycerophosphate, 1 mM DTT, 0.1 mM Na_3VO_4) in which glycerol concentration gradually increases from 10 % (top) to 30 % (bottom), 5.5 ml of a 10 % glycerol solution was mixed with 5.5 ml of a 30 % glycerol solution (11 ml total volume) in a centrifuge tube (Beckmann) using a gradient mixer. 3.5-10 mg of total protein extract was incubated with 0.04 % of NP-40 on a rotating wheel at 4ºC for 45 minutes and loaded onto the gradient. Then, the sample was centrifuged for 15 hours at 24500 rpm and 4 ºC using a SW-41TI rotor (Beckman). Nineteen 500-µl fractions were collected manually from top to the bottom. Gradients were calibrated using 11S catalase, 30S and 50S ribosomal subunits from *E. coli* and 60S subunits from *S. pombe*.

Precipitation of proteins with trichloracetic acid (TCA)

To precipitate proteins, 100 % (w/v) TCA solution was added into each 500 µl fraction from a glycerol gradient to a final concentration of 25 %. Next, samples were incubated at -20 ºC for 15 minutes and centrifuged at 13000 rpm for 45 minutes at 4 ºC. Then, the pellets were washed with 500 µl of ice-cold acetone and spun as above. After the samples were air dried for 15 minutes, the pellets were resuspended in 60 µl or in 100 µl of 1 × SDS sample buffer (2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerin, 100 mM β-mercaptoethanol, 100 mM Tris HCl, pH 6.8). Precipitated proteins were separated on SDS-PAGE and subsequently analyzed by western blotting.

Immunoprecipitation

For immunoprecipitation, antibodies bound to protein A sepharose (PAS) beads were used. 0.005 g of PAS beads were swelled in 50 µl of HB buffer for 1 hour at room temperature. Then, the PAS beads were washed twice in 50 µl of HB buffer and resuspended in 30 µl of HB buffer containing 2 % BSA and protease inhibitors (1 × Complete Mini EDTA-free, Roche Diagnostics, 4 µg/ml leupeptin, 1.3 mM benzamidin, 1mM PMSF). After 3 µg of appropriate antibodies were added, the beads were incubated with head-over-tail rotation for 2 hours at 4 ºC, centrifuged for 1 minute at 7000 rpm and 4 ºC, washed twice with 80 µl of HB buffer containing 2 % BSA and protease inhibitors and then resuspended in 30 µl of the same buffer. After a protein sample was added, the beads were incubated with head-over-tail rotation for 2 hours at 4 ºC and washed four times with 100 µl of IPP150 buffer (150 mM NaCl, 10 mM Tris HCl, 0.05 % NP-40, pH 7.5). For western blotting,

immunoprecipitate was resusupended in 20 µl of 2 × SDS sample buffer, incubated in a boiling water bath for 10 minutes and centrifuged. The supernatant was then separated by SDS-PAGE, immunoblotted and probed with appropriate antibodies as described. To probe for the presence of U snRNAs, from the immunoprecipitate RNA was isolated by phenol/chloroform extraction and ethanol/sodium acetate precipitation and used for RT-PCR analysis.

TAP tag purification of spliceosomal complexes

Prp31-CTAP containing spliceosomal complexes were purified from strain 714 (Table 5.2) as described by Gould *et al*. with several modifications of the protocol (Gould *et al*. 2004). Cells were grown in 5 L Edinburgh Minimal Medium (EMM) at 30 ºC in absence or presence of 200 mM thiamine and were harvested after 16–18 h. The cell pellet (~30 g fresh wet weight) was frozen in liquid nitrogen and stored at -80 ºC until use. As a negative control, strain 497, expressing HA-Prp31 and Myc-Prp1 was cultivated under the same growth conditions. For protein extract preparation, cells were disrupted in a bead beater (BioSpec Products) in 20 ml of NP-40 buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1 % NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 mg/ml leupeptin, 0.1 mM Na_3VO_4), containing protease inhibitors (1 × Complete Mini EDTA-free, Roche Diagnostics, 1.3 mM, benzamidin, 1 mM PMSF) and RNase inhibitor (20 mM vanadyl ribonucleoside complex), prepared after Berger and Birkenmeier (Berger S, Birkermeier C, 1979). After cell disruption, glass beads were washed with an additional 20 ml of NP–40 buffer. The lysate was cleared by centrifugation at 16000 x g for 15 min at 4 ºC and immediately used for purification. The extract was split into four aliquots of 10 ml and placed into 0.8 × 4 cm chromatography columns (Bio-Rad, Poly-Prep), each supplied with 300 µl bed volume of IgG-Sepharose beads (GE Healthcare, IgG-Sepharose 6, Fast Flow) equilibrated in NP-40 buffer.

The beads were incubated on a rotating wheel at 4 ºC for 2 h and subsequently washed three times with 10 ml of IPP150 buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1 % NP-40) and three times with 1 ml of TEV cleavage buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1 % NP-40, 0.5 mM EDTA, 1.0 mM DTT). After the last washing step, the beads were resuspended in 300 µl of TEV cleavage buffer. Then, 40 U recombinant TEV protease (MoBiTec GmbH, Goettingen) were added and the beads were incubated on a rotating wheel at 18 ºC for 3 h.

After incubation with TEV protease, about 1300 µl of the eluate were collected. Three-hundred microliters of the eluate were mixed with 100 ml of 100 % TCA and the precipitated proteins were analyzed by western blotting and Coomassie staining. To the remaining eluate (about 1000 µl) 3 ml calmodulin binding buffer (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % NP-40, 1mM DTT) were added and incubated with 300 µl calmodulin affinity resin (Stratagene) on a rotating wheel for 1 h at 4 ºC. The beads were washed with 3 ml calmodulin binding buffer containing 0.1 % NP-40 and 2 ml calmodulin binding buffer containing 0.02 % NP-40. The resin bound complexes were eluted either under native conditions by resuspending the resin in 1000 µl calmodulin elution buffer (10mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.02 % NP-40, 20 mM EGTA, 1mM DTT) or denaturing conditions using calmodulin elution buffer containing 0.1 % sodium dodecyl sulfate (SDS).

About 300 µl of the eluate were used for TCA precipitation. Precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequent western analysis and/or Coomassie staining. From the remaining eluate (700 µl), RNA was isolated by phenol/chloroform extraction and ethanol/sodium acetate precipitation.

Abbreviations

ade	adenine
amp	ampicilin
ars	autonomously replicating sequence
bp	base pair
DNA	deoxyribonucleic acid
DNase	deoxyribonucease
dNTP	2′-deoxy-nucleoside-5′-triphosphate
DTT	1,4-dithiothreitol
EDTA	ethylenediamine-tetraacetat
g	gramme (mass)
h	hour (time)
HA	influenza hemagglutinin peptide
kb	kilobase
kDa	kilodalton
L	liter
lac	lactose
leu	leucine
M	mol/l (molarity)
m	milli
min	minute (time)
Myc	human p62-c-Myc peptide
nmt	no massage in thiamine
ºC	degrees Celsius
OD	optical density
ori	origin of replication
pH	negative logarithm (base 10) of the molar concentration of hydronium ions
prp	pre-mRNA processing
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
s	second (time)
SDS	sodium dodecylsulfate
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein particle
TBE	Tris-Borate-EDTA-buffer
TBS	Tris buffer saline

TPR	tetratricopeptide repeat
Tris	tris(hydroxymethyl)aminomethane
U	unit (enzymatic activity)
ura	uracil
UV	ultraviolet
V5	paramyxovirus SV5 peptide found in the P and V proteins
V	volt (voltage)
v/v	volume/volume
w/v	weight/volume
F	farad (capacitance)
Ω	ohm (electrical resistance)
µ	micro

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Aknowledgements

Die vorliegende Arbeit wurde im Institut für Genetik, TU Braunschweig, in der Arbeitsgruppe B (Käufer) angefertigt.

I would like to thank the members of my PhD committee Prof. Dr. Norbert F Käufer, Prof. Dr. Hans-Henning Arnold and Prof. Dr. Petra Dersch for reviewing this work and giving me an opportunity to defend it.

Special thanks go to Prof. Dr. Norbert F. Käufer for giving me an opportunity to work in his group, his excellent guidance, many hours of interesting and vibrant discussions, for his passionate attitude towards since and especially his help in preparation of this manuscript.

I would like thank Dr. Martin Lützelberger for his scientific support, useful technical tips, prolific discussions and his help in preparation of this manuscript.

I would like to thank Susanne Zock-Emmenthal and Ulrike Brandt for their excellent technical support and kind attitude and also my other colleges for creating nice working environment in the laboratory. I would like to thank Marion Utecht and Ute Kyrath for their administrative assistance.

I would like to express my gratitude to the organizers of the International Graduate College "Molecular Complexes of Biomedical Relevance" at the Technical University in Braunschweig Prof. Dr. Stefan Dübel, Prof. Dr. Jürgen Wehland, Prof. Dr. Brigitte M. Jockusch, Dr. Annika Guse and Dr. Manuela Schüngel and to the government of the federal state Niedersachsen for three years of funding.

I would like to thank Nils Hahn for interesting discussions and for his help in the isolation of the *spp101-1* suppressor.

Finally I would like to thank my Family, especially my Mother and my Grandmother for their unconditional love, help and understanding. And all people who helped me during my PhD work.

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